ULTRASTRUCTURAL STUDIES OF NEUROSECRETION IN *PERIPLANETA AMERICANA*, L.

with special reference to the optic lobe and to ancillary circulatory structures

Volume I

by

TERENCE MICHAEL BEATTIE, B.Sc (Tas.),
B.Sc. Hons (Tas.)

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T. M. Beattie.

T. M. BEATTIE.

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ABSTRACT

This thesis presents mainly ultrastructural data on neurosecretory (NS) cells in the optic lobe and on several ancillary circulatory structures.

A vital staining technique for NS material using acridine orange is described. All known types of NS material in insects give a positive reaction with this technique.

The ultrastructure, histology and histochemistry of a new group of NS cells in the optic lobe are described. The axons of these cells are innervated by axons containing small dense granules which probably control the function of the NS cells. The optic lobes have been previously postulated as the site of a circadian clock controlling locomotor activity and the NS cells may be intimately involved with this clock mechanism. Initial experiments to find a causal relationship between the NS cells and activity were equivocal.

Four pairs of segmental blood vessels occur in association with abdominal excurrent ostia of the heart. The segmental blood vessels are composed of fibroblasts and connective tissue strands and have numerous longitudinal NS axons which form an extension of the neurohaemal organ associated with the lateral cardiac nerve. The number of release sites in the NS axons show a marked diurnal

variation.

The valves of the segmental blood vessels are composed of a peculiar type of muscle. They are innervated by granule-containing axons. The valves have a rhythm different to the heart, and it appears as if they have different controlling mechanisms. The valves appear to control the distribution of blood to various regions of the abdomen.

The NS axons of the lateral cardiac nerve are at least partly derived from segmental nerves of the ventral cord as well as from the median/transverse nerves. There is a pair of peripheral NS cells at the junction of the median/transverse nerve and the link nerve. There is also a multipolar neuron in this region. The link nerve appears to constitute a neurohaemal site.

Accessory pulsatile organs associated with the antennae have a complex structure. Briefly, two ampullae are attached to the frons and they are connected to each other by a pulsatile muscle. There is a neurohaemal system in the ampulla wall which contains terminal Herring bodies. A vessel runs to the antenna from the ampulla. The cells of the vessel wall are specialized and have many resemblances to other epithelia involved in active transport of ions and/or water.

GENERAL INTRODUCTION

Periplaneta americana has been used for many types of investigation. Its large size, ready availability and ease of culture make it a popular and useful laboratory animal. Much of the biology of P. americana as well as other cockroaches has been recently summarized by Guthrie and Tindall (1968), and it is apparent that most systems in this animal have received at least some attention.

In this thesis, aspects of the nervous system and the circulatory system are investigated. As in many systems, structure forms the basis for further physiological investigation. The major portion of the thesis is based on ultrastructure, but much of the interpretation is from a physiological viewpoint.

For this work, neurosecretory cells will be defined as those neurons which show cytological evidence of glandular activity. This concurs with Ernst Scharrer's concept of neurosecretion and a hormonal definition is not required (B. Scharrer, 1969), although many types of neurosecretory material appear to have hormonal properties. Many of the investigations of neurosecretory phenomena in invertebrates are of a cytological nature and only in a few cases has there been any physiological investigation (Maddrell,

1967, 1969). However, from the few well investigated cases, it would appear that neurosecretory cells have a profound influence on many physiological functions.

Staining techniques, such as paraldehyde fuchsin or chrome haematoxylin/phloxin, stain some neurosecretory materials selectively, but other neurosecretory materials can only be stained with more general techniques such as Heidenhain's azan. In Chapter 1 a staining technique is described which appears to stain all types of neurosecretory material in P. americana and other insects and invertebrates. The technique involves vital staining of tissue with acridine orange. With blue light fluorescence, the neurosecretory material stains metachromatically and is distinct from other elements of the nervous tissue. This method is useful for identifying neurosecretory material in relatively thin pieces of tissue.

The optic lobes of insects contain a series of intricate neuropiles which function as integrative centres not only for visual information but also for other sensory inputs from all parts of the body (Horridge, 1968). Crustaceans have a complex neurosecretory system associated with the optic lobe (Gabe, 1966), but its relation to sensory input information is largely unexplored. In insects, the cephalic neurosecretory system has previously been thought to consist only of the median pars intercerebralis perikarya as well as some lateral perikarya which send processes to the

corpora cardiaca. Apparently no neurosecretory system was known to be present in the optic lobe although, by analogy with crustaceans, this might well have been expected. Chapter 2 describes part of a neurosecretory system in the optic lobe of Periplaneta; the terminations of the axons were not located. The neurosecretory material in this system does not stain with the so-called neurosecretory stains, but can be visualized with Mallory's triple stain and Heidenhain's azan. Ultrastructurally, the neurosecretory cells have features characteristic of actively synthesizing cells and the elementary granule product is accumulated to varying degrees in the cytoplasm. The histochemistry of these cells was also investigated.

The function of this optic lobe neurosecretory system is not known although an attempt was made to show a relationship between it and circadian rhythms (Chapter 3), since the optic lobes appear to be the site of the "biological clock" controlling locomotor activity (see review by Brady, 1969).

Despite its importance, the circulatory system of insects has not received a great deal of attention from a structural approach in recent times. The gross anatomy of the heart of many insects has been described (see review by Jones, 1964), but ultrastructural studies are few (e.g. Edwards and Challice, 1960; Bacetti and Bigliardi, 1969; Sanger and McCann, 1968). Similarly, the innervation of

the heart of the cockroach was described many years ago (Alexandrowicz, 1926) and only recently has any further structural investigation been made using modern techniques (e.g. Johnson, 1966a; Miller and Thomson, 1968). Chapter 4 gives a description of the ultrastructure of the abdominal segmental blood vessels. The walls of the vessels contain numerous neurosecretory axons which arise from the lateral cardiac nerve and form extensions of the cardiac neurohaemal organ. The vessel walls are similar to the dorsal diaphragm in that they are composed of fibroblasts and connective tissue strands.

Chapter 5 gives a description of part of the peripheral nervous system which forms the pathway innervating the heart. The lateral region of the nervous system is quite complex and has a neurosecretory system associated with it. A similar system has been described by Finlayson and Osborne (1968) in Carausius morosus. During the course of the work on the peripheral nervous system, three pairs of imaginicaducous muscles were found in the lateral regions of the abdominal segments. The anatomy and degeneration of these muscles in described in Chapter 6.

Circulation of haemolymph in appendages is usually aided by accessory pulsatile organs (accessory hearts). These organs have a variable form depending on the anatomical location and the species of insect (Jones, 1964). For circulation in the antennae of Periplaneta, there is an

ampulla at the base of each antenna and these are connected to each other by a pulsatile muscle. A vessel leaves each ampulla and runs down the antenna. The basic morphology of this system was described by Pawlowa (1895). In Chapter 7 the ultrastructure of this system is described. The pulsatile muscle contains two types of muscle fibre and has numerous granule-containing axons associated with it. Some of the axons form a neurohaemal organ in the ampulla wall beneath the pulsatile muscle insertion. The neurohaemal organ contains large Herring bodies which show signs of degeneration. This is unusual for insect neurohaemal organs. The walls of the ampullae have well developed connective tissue layers and these are probably involved in the pumping action of the ampullae. The initial convoluted portion of the antennal vessel has a complex structure and has morphological characteristics of epithelia involved in active transport of ions and/or water. The distal portions of the antennal vessels (i.e. those parts within the antennae) do not show these structural specializations and appear to act as conduits for haemolymph.

The thesis has been arranged in two volumes to expedite correlation of the text (Volume I) with the illustrations (Volume II). A few Text Figures are included in Volume I. The illustration numbers in Volume II are prefixed with the Chapter number of Volume I.

VITAL STAINING OF NEUROSECRETORY MATERIAL WITH ACRIDINE ORANGE

INTRODUCTION

Vital staining of fixed cells with acridine orange produces an orthochromatic green fluorescence in nuclei and sometimes a metachromatic red fluorescence in cytoplasmic granules. It is now generally accepted that green nuclear fluorescence is due to nucleic acids and the red cytoplasmic granules are lysosomes (Allison and Young, 1964; Koenig, 1963; De Bruyn et al., 1953; Novikoff, 1967; Robbins and Marcus, 1963; Robbins et al., 1964; Zelenin, 1966, however see Austing and Bishop (1959) for an alternative interpretation). As well as staining with acridine orange, lysosomes show acid phosphatase activity amongst other hydrolytic enzymes (Allison and Young, 1964; De Bruyn et al., 1953; Koening, 1963; Novikoff, 1967).

Neurosecretory (NS) materials vary in their chemical composition between different NS cells and no one staining technique has been found to stain all types of NS material (Gabe, 1966). In this chapter, it is shown that acridine orange can be used as a vital stain for all NS materials. This work has been published (Beattie, 1971a).

MATERIALS AND METHODS

Adult and larval forms of Periplaneta americana from laboratory colonies were used in this study. The following stains were obtained from Chroma-Gesellschaft;

acridine orange, acridine yellow, coriphosphine, acriflavine, phosphin 3R, euchrysin 3RX, and euchrysin 2G. All are acridine derivatives. They were dissolved in 0.9% NaCl at a concentration of 0.1 mg/ml.

Pieces of nervous tissue were dissected from the insect under saline and placed in a drop of stain solution on a microscope slide. The tissue was stained for 1 minutes and then moved to a drop of saline further along the slide. A coverslip was added and excess saline removed. The whole mount was then viewed with blue light from a Wild microscope fluorescence system.

For acid phosphatase localization, tissue was fixed in formol-calcium over-night. The Gomori lead method and the simultaneous coupling azo dye method using naphthol AS-TR and hexazotized pararosaniline were used (Barka and Anderson, 1963).

The stains were analysed by thin-layer chromatography using kieselgel and n-butanol:ammonia:ethanol:water (16:0.15:5:5) as developing solvent (Kasten, 1967). The chromatograms were viewed with long wavelength UV-light.

RESULTS

Differentiation of NS cells and "ordinary" nerve cells was obtained with acridine orange, euchrysine 3RX and coriphosphine, however the latter showed only weak differentiating ability. Negative results were obtained with the other stains. NS material appeared as red fluorescing

granules which were probably lysosomes. Also, the axons of these cells contain considerable numbers of small red granules. The nerves to the corpora cardiaca show red granules in some axons and only weak green general staining in others. The corpora cardiaca possessed scattered red granular accumulations among the intrinsic cells whose nuclei stained green. This distribution of NS material agrees with electron microscope studies made by Scharrer (1963). Nerves leading to, around, through, and away from the corpora allata all exhibit accumulations of red granules. Those axons passing through the corpora allata often had a varicose arrangment of red granules (Fig. 1-1). Again, this distribution agrees with electron microscope studies made by Scharrer (1964). Also, the median nerves of the ventral nerve cord exhibit red granules in some of the axons proximal and distal to the neurohaemal organs contained therein (De Bessé, 1966; Brady and Maddrell, 1967).

Since it was thought that NS material in other animals may give a similar reaction with acridine orange, the following representative systems which contain NS material were investigated; the eye stalk and pericardial organ of the crab, Paragrapsis gaimardii, the retrocerebral system of Calliphora stygia, the heart of Helix aspersa, and the cerebral ganglia of the earthworm, Megascolides polynephricus. In each case, NS cells and/or axons could be distinguished from other nerves and axons.

Acridine orange and euchrysin 3RX gave similar chromatograms which showed 6 (possibly 7) components with the slowest moving spot being the major constituent. The chromatogram of coriphosphine showed a fairly strong spot with an Rf value and fluorescent colour similar to the major component of acridine orange, but the other spots were all different in Rf value and/or fluorescent colour.

Acid phosphatase was not localized in either type of axon in the lateral cardiac nerves by either of the histochemical methods used.

Unstained whole mounts of the lateral cardiac nerves were viewed under dark field illumination. NS material exhibited its characteristic blue colour in the form of granules or dense accumulations. The distribution of NS axons revealed by this method was similar to the distribution shown by the vital staining with acridine orange.

DISCUSSION

The metachromatic red fluorescence of NS material when stained with acridine orange allows a rapid method for identifying NS cells and axons. All the known sites of NS material in the insect nervous system give positive results by this method whereas different staining techniques and electron microscopy have been necessary to show the presence of all types of NS material. A similar staining method (Zeiger and Harders, 1951) has been used on the nerves in the

mesentery of the frog, Rana temporaria and the cerebral ganglia of Lumbricus terrestris and it was found that the nerve cell bodies and axons contain red granules. Some of the granules have a varicose arrangement in the axons. This, together with the positive reaction in the NS cells and axons of the other animals used in this study, would indicate that all NS and other membrane-bound materials have some common feature which makes them stainable with acridine orange. Whether this a particular chemical compound or a particular type of membrane structure which combines with the acridine orange is not known. However, the entities responsible probably possess a highly oriented array of negative binding sites which are spaced at a distance which allows interaction between the adjacent acridine orange molecules (Kasten, 1967).

The absence of acid phosphatase in the NS axons would suggest that it is not the presence of lysosomes which produce the metachromatic staining, but that the NS material itself takes up this stain. Whether this similarity in staining is due to similar compounds or membranes is unresolved, but both lysosomes and NS granules contain biologically active substances in a latent form (Gabe, 1966; Novikoff, 1967).

The thin-layer chromatographic analysis of the three stains which gave positive results indicate that acridine orange and euchrysine 3RX are the same, or very similar, products and that coriphosphine is contaminated with acridine

orange. It is this material which gives metachromatic staining of NS material.

HISTOLOGY, HISTOCHEMISTRY AND ULTRASTRUCTURE OF NEUROSECRETORY CELLS IN THE OPTIC LOBE

INTRODUCTION

The occurrence of neurosecretory neurons in the nervous system of insects is widespread but the function of many of these neurons has not been elucidated (Maddrell, 1967). Cytological evidence is, however, the usual starting point in any investigation of neurosecretory systems and serves as a basis of physiological studies. The staining properties of neurosecretory material have been shown to be diverse and not all types give a positive reaction with the basic stains, paraldehyde fuchsin and chrome-haematoxylin, but may react with acid stains. Perhaps more indicative of neurosecretory cells is the production of elementary granules by the Golgi bodies in the perikaryon when the tissue is studied at the ultrastructural level.

The occurrence and distribution of neurosecretory cells in the brain of insects is well documented (Gabe, 1966). In the case of Periplaneta americana, Willey (1961) has described groups of cells in the pars intercerebralis which give rise to the nervi corporis cardiaci I and II. Furthermore, Khan and Fraser (1962) have described median, lateral and ventral groups of neurosecretory cells containing paraldehyde-fuchsin-positive granules. Little work has been done on PAF-negative neurosecretory cells in Periplaneta

americana although there has been several recent histochemical studies on this type of cell in other insects (Ramade, 1969; Raabe and Monjo, 1970).

The ultrastructure of neurosecretory cells in the pars intercerebralis of Blaberus craniifer has been studied by Willey and Chapman (1962). They found that the neurons contain granules up to 150nm in diameter associated with the Golgi bodies. Similarly, Bern et al., (1961) found electron-dense granules of about this size in the neurosecretory cells of P. americana. The cells in both these studies are most probably PAF-positive.

There has been no previous report of neurosecretory cells in the optic lobe of insects which is in contrast to the situation in crustaceans (Gabe, 1966). In this chapter the histology, histochemistry and ultrastructure of a new group of neurosecretory cells in the optic lobe of Periplaneta americana is described.

MATERIAL AND METHODS

For light microscopy, whole brains with optic lobes were dissected from adults and larvae and fixed in Bouin's fluid. 6µ sections were stained with paraldehyde fuchsin (Ewen, 1962), or with a modified azan technique (Hubschman, 1962), or with Mallory's triple stain (Patin 1946). The following histochemical tests were carried out:- mercury bromophenol blue for proteins (Mazia et al., 1953), PAS for 1,2-glycols, DMAB-nitrite for indol derivatives (Adams, 1957), diazo-safranin for 5-hydroxytryptamine

and related substances (Lillie et al., 1953), the Liebman method for arginine (Liebman, 1951), and the alcian blue/alcian yellow technique for acid groups (Peute and Van Der Kamer, 1967).

For electron microscopy, optic lobes were fixed for 1 hour in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.3) and post-fixed in 2% OsO_4 in buffer. Epon embedded tissue was sectioned and stained with uranyl acetate and lead citrate.

RESULTS

The first indication that there may be neurosecretory cells in the optic lobe of Periplaneta americana was the observation that two small patches of bluish tissue could be seen on the posterior aspect of each optic lobe when the head was dissected. This Tyndall effect, which is characteristic of neurosecretory material, prompted further investigation.

The neurosecretory cells form two flattened cone-shaped groups on the posterior face of the optic lobe immediately beneath the perineurium in the region of the chiasma between the lamina ganglionaris and the medulla terminalis (Figs. 1a, 1b). The two groups of cells are usually separated from one another, but occasionally there is a thin strand of cells connecting them. Because of the similarity of the two groups of cells, it was initially thought that there was only one group which tended to be variable in form. It now appears as if the groups are virtually sep-

arate, one group being dorsal and the other ventral to axis running lengthwise through the optic lobe. The size and staining characteristics of the cell in each group appear to be identical. Also, at the ultrastructural level, there are no obvious differences. The following description applies equally well to both groups of cells.

There are about 120 monopolar cells in each optic lobe (i.e. total for both groups), and they are usually ovoid to pyriform in shape. The cells measure about 16/um through the major axis and 9/um through the minor axis. Nuclei are round to ovoid and may measure 10/um to 6/um. The neurosecretory cells are larger than the surrounding neurons and possess a greater proportion of cytoplasm. Sometimes, an axon hillock can be identified. In these latter cases, the axon runs towards the centre of the distal optic chiasma (Fig. 1c).

The neurosecretory material (NSM) does not stain with paraldehyde fuchsin, but it does react with the light green component of the counterstain. With the azan technique, NSM is stained either red or sometimes deep blue. Often the entire cytoplasm is filled with stainable material, but in other cases, the NSM occurs as isolated patches in the cytoplasm. The histochemical tests showed that the NSM is positive for proteins, negative for 1,2-glycols, and contains no strong acidic groups which may have been derived from S-S or S-H groups. Also, there was only a slight

reaction for weakly acidic groups. The DMAB- nitrite reaction was positive, indicating the presence of an indol derivative and this is most probably ~~trytaphan~~ tryptophan since the diazo-safranin reaction was negative. Arginine is present in high concentrations in some of the cells whereas in others only a moderate amount of this basic amino acid is present. When viewed with phase contrast optics, the cells rich in arginine show very granular nuclei whereas the other neurosecretory cells have almost clear nuclei.

At the ultrastructural level, the neurosecretory cells contain masses of elementary granules (Figs. 2,3,4,6). These are electron dense and the majority have a diameter in the range 100-170nm. The shape of the granules is spherical to ovoid. The number of granules seems to vary between individual animals. In some, the granules fill every available space; in other animals, they are scattered more sparsely amongst the other cell organelles. Rough endoplasmic reticulum is usually fairly well developed and the cisternae often form concentric layers around the nucleus. There appears to be some correlation between the number of granules and the degree of development of the endoplasmic reticulum:- large numbers occurring with good organization and a fewer number occurring with poor organization. In the few larval animals studied, there appear to be fewer elementary granules than in adult animals.

Ribosomes stud the membranes of the endoplasmic

reticulum but also occur free in the cytoplasm either singly or in groups. Ribosomes on the endoplasmic reticulum appear to be arranged in circles or spirals (Fig.7a). This is best seen in tangential sections of the endoplasmic reticulum. There are about 10 ribosomes in each formation. This arrangement accounts for the apparent discontinuous distribution of ribosomes on the membranes when the endoplasmic reticulum is sectioned perpendicularly. At the places where there is an accumulation of ribosomes on the endoplasmic reticulum, the material between the membranes is slightly more electron-dense. This may indicate a local accumulation of protein at the site of synthesis.

Golgi bodies are well developed and show a marked polarization in their structure (Figs. 5a, 5b). The cisternae contain electron-dense material and occasionally an almost complete elementary neurosecretory granule can be seen at the periphery of one of the cisternae. The forming face of the Golgi body is associated with large irregular shaped membrane profiles which are apparently continous with the endoplasmic reticulum since scattered groups of ribosomes are sometimes found on the membranes of these structures (Fig. 5b).

Dense bodies, which probably represent autophagic lysosomes or lipofuscin granules, are a regular feature of these cells (Fig. 5c). They are up to 1 µm in diameter and occur in any part of the cytoplasm. Internally, they con-

sist of electron-transparent units and multilaminar units, both of which are set in an electron-dense matrix.

The nuclei have sparsely scattered chromatin and possess one or two nucleoli (Fig. 6). The nuclear membrane is usually irregular in outline and has numerous nuclear pores (Figs. 5a, 6).

Ovoid regions composed of granular or fibrous material with no limiting membrane are sometimes found in the cytoplasm of the cells (Figs. 4, 7a, 7b). The regions are 2-3 μm along the major axis. The centre of the regions invariably contain neurosecretory granules, ribosomes and mitochondria. Neurosecretory granules, ribosomes and rough endoplasmic reticulum are also scattered throughout the rest of the regions. The occurrence of these regions is not restricted to any particular part of the cytoplasm. However, most of them have one or two Golgi bodies close by and neurosecretory granules usually form a perimeter. No obvious structural connection could be found between them and any other cell organelles.

Some of the neurosecretory cells have a more densely staining cytoplasmic matrix than other neighbouring neurosecretory cells (Fig. 6). There is no apparent difference between the organelle complements of the two different staining cells.

Each neurosecretory cell is separated from adjacent nerve cells by one or two layers of glial cell process.

This glial cell layer varies from 0.1 /um to 1 /um in thickness. The plasmalemma of the neurosecretory cells often shows invaginations which are filled with glial tissue (Figs, 3, 4). The invaginations are usually 1-2 /um deep and ca. 0.5 /um wide. The cytoplasm of the glial tissue filling the invaginations shows no special features and does not appear to be markedly different from the rest of the glial tissue. On the other hand, the neurosecretory cell cytoplasm quite often has elements of endoplasmic reticulum closely associated with the invaginations as well as other regions of the plasmalemma.

The axons of the neurosecretory cells are about 1 /um diameter and pass between the cell bodies as they run towards the interior of the optic lobe (Figs. 4, 6). A short distance from the cell bodies, the axons become intermingled with another type of axon containing small electron-dense granules about 70nm in diameter (57-93nm range). Synapses of these latter axons onto the axons containing large neurosecretory granules have been found (Figs. 8b, 9a, 9b). Groups of synaptic vesicles (44nm mean diameter, 37-56nm range) occur in the presynaptic axon, often intermingled with the electron-dense granules. The thickened membranes of the synaptic junction are parallel and are spaced ca. 20 nm apart. There is a plate of dense material in the synaptic cleft and a sub-synaptic web is always present at each synapse. In the axon tracts, branching

axons are sometimes found. Alternatively, one of these branches may represent a dendrite or a collateral. Sometimes, there is a synapse on one of the branches (Fig. 9c).

After passing through the synaptic region, the neurosecretory axons cross over to the other side of the optic lobe at the distal optic chiasma and run towards the brain. The bundle of neurosecretory axons in this region is about 10 µm in diameter. Numerous neurotubules are present in the axons and the neurosecretory granules tend to occupy the peripheral portions of the axoplasm (Fig. 10). Throughout the axoplasm of both granule-containing fibres there exists a ramifying system of tubular endoplasmic reticulum (Figs. 8b, 9a, 9b, 10). The diameter of the tubules varies from 40 nm to 60 nm. This is considerably larger than the diameter of the neurotubules (20-22nm) and there appears to be continuity between these two structures. Branches of this sytem often appear to be associated with mitochondria and neurosecretory granules. This association may be merely fortuitous or may represent a functional configuration. In the synaptic regions, this tubular system is present (Figs. 8b, 9a, 9b), but does not appear to give rise to synaptic vesicles.

Along the course of the neurosecretory axons, groups of small vesicles are occasionally found associated with the axolemma. There is a plaque of dense material immediately adjacent the membrane beneath the vesicle cluster. This type

of structure is characteristic of probable release sites of neurosecretory material (Fig. 8b). From these observations, en passent release sites are possible. The important point is that at these sites the neurosecretory material appears to be released within the nervous system and not close to a vascular element or stromal material which communicates with the haemolymph.

The cross-sectional area (directly related to volume) of the neurosecretory cells and their nuclei show variation throughout a 24 hour period. Both cell area and nuclear area show concurrent variation. Two maxima are shown in a daily cycle. The greater peak occurs at, or just after, the light-off time, and the smaller peak prior to the light-on time. These two peaks are temporally related to the peaks of locomotor activity. The maximum area (volume) occurs a short time before the onset of the main activity peaks. This will be discussed further in a later chapter.

There are two other groups of cells in the optic lobe which are interesting. For the sake of convenience, they will be reported at this stage.

A small group of granule-containing nerve cells are present at the proximal optic chiasma. These cells appear to be neurosecretory (Figs. 11, 12). The cells are ca. 20/um in diameter and have a centrally placed ovoid nucleus (ca. 7/um by 10/um). In the cytoplasm, there are

many membrane-bound dense granules. They have a mean diameter of 87nm and a range of 43-147nm. The granules are aggregated into clumps in all regions of the cytoplasm. The rough endoplasmic reticulum does not have a well organised arrangement, and usually short sections are found throughout the cytoplasm. There are many polyribosomes. The numerous Golgi bodies appear to be quite active, budding off dense granules from the ends of the cisternae. There are quite a few dense bodies with vacuolar and membranous contents. These are similar in structure and size to those found in the other groups of neurosecretory cells at the distal optic chiasma. Also, there are occasional multilaminar bodies, some of which appear to be associated with mitochondria. The plasmalemma is undulating and has occasional invaginations. Several layers of glial tissue surround each cell. Although the axon hillock was found in some cases (Fig. 12), the axon tracts were not located.

The other group of interesting cells appear to be a special type of glial tissue. They form crescent-shaped groups of dense cells on the anterior aspect of each optic chiasma in the optic lobe (Figs. 1, 13). At the distal chiasma, the crescent runs from the dorso-posterior area to the ventro-posterior area. The cells are close to the axon tracts and are immediately median to the lamina ganglionaris. They have an overlying layer of neurons and glia on the peripheral aspect. The cells are elongate,

measuring ca 20/um by 3/um. The nuclei are similarly shaped, measuring 10/um by 2/um. The long axes of the cells are all oriented in the same direction, and this is parallel with the long axis of the optic lobe.

At the light microscope level, the cells form a compact mass. The cytoplasm is acidophilic, even after permanganate oxidation. With PAF, the cytoplasm is strongly stained green and contains small bright orange granules. The cytoplasm is a granular purple red when azan is used and a strong red with Mallory's. Histochemically, the cytoplasm is positive for proteins and 1,2-glycols. In the latter test, strongly stained granules could be found in the cytoplasm.

At the ultrastructural level, the cells have dense cytoplasm. This density is due partly to the large number of ribosomes and partly to the overall density of the cytoplasmic matrix. Quite often, the cytoplasm contains large masses of clustered glycogen granules (Figs. 13, 14b). The plasmalemma at the "exposed" surfaces is often highly infolded to produce fine cytoplasmic projections (Fig. 14c). The membranes of the terminal portions of the projections are strongly stained. The cell membranes of adjacent cells have desmosomes between them here and there along the boundaries (Fig. 14a). Centrioles are often found in these cells (Figs. 13, 14c).

At the proximal optic chiasma, there is a similar

group of cells. This group is not as large as the distal group, but occupies the same general position. The cells of the proximal group have the same staining characteristics as those of the distal group. Their ultrastructure has not been investigated, but it is probably essentially similar to the distal cells.

These two groups of cells have some similarities to glial tissue (e.g.storage function, Wigglesworth, 1960), but the reason why they should occur as distinct compact masses is intriguing. The cells do not appear to be associated with any other particular tissue. Also, their consistent occurrence on the anterior side of the optic chiasmata may indicate a functional relationship with these portions of the axon tracts from the eye.

DISCUSSION

The neurosecretory cells in the optic lobe of Periplaneta americana are yet another example of neurosecretory cells which do not stain with paraldehyde fuchsin, but remain acidophilic after permanganate oxidation. The variable staining reaction with the azan technique may be due to slight variations in the staining and differentiating times, but could also be influenced by cyclical activity, (this is discussed further in a later chapter). The red staining by azocarmine is the more usual situation in acidophilic C-cells of insects (e.g. Raabe, 1965). The affinity of NSM for the aniline blue counterstain has not

been reported in insects except for the violet aggregates in the perisympathetic neurohaemal organs of the ventral nerve cord of phasmids (Raabe, op. cit.).

The contention of Brady and Maddrell (1967), that acidophilic NSM of C-cells corresponds with electron-transparent granules and that PAF-positive NSM corresponds with electron-dense granules does not hold for the NSM in the cells of the optic lobe of the cockroach. In this case, acidophilia occurs with electron-dense granules 100-170nm in diameter. A similar situation exists in several other insects. For example, in Musca domestica, C-cells have been shown to contain electron-dense granules about 150nm in diameter (Ramade, 1969), and in Locusta migratoria, the C-cells contain dense granules 100-200nm in diameter (Girardie and Girardie, 1967). From these different cases, it would appear that there is no uniformity in either electron density or in diameter of elementary granules in acidophilic neurosecretory C-cells.

The variable amount of NSM stainable with azan technique may indicate different stages in the secretory cycle or that the cells are a heterogenous group of cell types. All the cells stain the same colour and show variations in staining intensity and not variations in the type of staining reaction in any one animal. Furthermore, the neurosecretory cells show a marked variation in size, depending on the time of day. A proportion of the cells do show

a reduced amount of stainable material when the cells are near their maximum size. However, at the ultrastructural level, adjacent cells sometimes have different degrees of staining intensity. This may be interpreted as either different types of cells, or as cells which are synthetically out of phase with each other. The histochemical test for arginine is interesting in this respect in that the nuclei of strongly positive cells are very granular and the weakly staining cells have almost clear nuclei. The granular nuclei appear to be associated with synthetically active cells whereas clear nuclei appear to be associated with less active or inactive cells. This may again indicate some sort of cyclical activity in these neurosecretory cells.

There have been several histochemical studies on acidophilic neurosecretory cells in insects. In the C-cells of the phasmid Clitumnus extradentatus, the NSM is a protein rich in tryptophan (Raabe and Monjo, 1970). Baudry and Baehr (1970) working on Rhodnius, found that the protocerebral and sub-oesophageal C-cells are positive for tryptophan but not the C-cells of the ventral nerve cord. The NSM of the neurosecretory cells in the optic lobe of Periplaneta is likewise a protein rich in tryptophan but also contains considerable amounts of the basic amino acid, arginine. This latter fact probably accounts for the histological staining reaction of the NSM.

At the ultrastructural level, these cells show

all the characteristics of secretory neurons. The well developed endoplasmic reticulum, the prominent Golgi bodies and the accumulated masses of elementary neurosecretory granules indicate that the cells are synthetically active. The increased electron density of the more mature cisternae of the Golgi bodies indicate that the contents are in a more condensed state and the cells are actively producing NSM (Scharrer and Brown, 1961). The apparent continuity between the endoplasmic reticulum and the Golgi bodies is supported by similar observations on other tissues (e.g. Morré et al., 1970). The secretory material appears to be enclosed first within the cisternae of the endoplasmic reticulum, then the membranes of the Golgi bodies, and finally within the granule membranes (see Beams and Kessel, 1968). At every stage the proteinaceous material is contained within the same membrane, but it is probably modified during transition.

The number of dense bodies or lipofucsin granules is probably related to the age of the animal (Peters et al., 1970). These granules are thought to be derived from lysosomes and represent accumulations of degraded material as a result of "wear and tear" during the life of the cell. The increase in number of these granules in adult animals as compared with larval stages concurs with the aging hypothesis.

Similar structures to the ovoid regions composed

of granular and fibrous material have been reported previously in neurosecretory and other neurons (e.g. Le Beux, 1971: Le Beux et al., 1971). Although these regions have some similarities to the nucleoli, they are thought to be distinct entities unrelated to nucleoli. Le Beux has shown that a system of microfilaments fan out from these regions and form links with ribosomes, cytoplasmic membranes and the plasmalemma thus forming a filamentous network. Also, neurofilaments were shown to be present in the core of these structures. The equivalent structures in Periplaneta appear to be associated with all cellular organelles and do not show any particular relationship with any of them. These structures do not appear to have been previously reported from insect nervous tissue. Functionally, they are unexplored and morphological evidence is not particularly helpful in predicting a function for these regions.

The glial-filled invaginations of the plasmalemma of the neurosecretory cells are possibly associated with the supply of nutrient. The avascular nervous system of insects must have a mechanism for the transfer of nutrients from the haemolymph to the metabolically active nerve cells (Smith, 1968; Kandel and Kupfermann, 1970). The glial tissue of Periplaneta has extensive stores of glycogen and lipid (Wigglesworth, 1960), and it is probably transferred to the nerve cells on demand. The endoplasmic reticulum of the

nerve cells is most likely involved in this process since it has an intimate association with the plasmalemma apposed to the glial tissue (Smith, 1968).

Since there are two groups of neurosecretory cells at the distal optic chiasma, the origin of neurosecretory axon tracts in the more proximal parts of the optic lobe must be queried. Certainly, these tracts arise from the neurosecretory cells and cross over at the distal chiasma. The situation becomes more difficult to interpret at the proximal chiasma because one is not really certain which tracts belong to each group of cells. This is mainly a technical problem. Serial thin sectioning over any length of tissue is difficult to obtain, so the question of identity remains unresolved. Another complicating factor is the occurrence of a further group of neurosecretory cells at the proximal chiasma. Although the diameter of the granules in these cells is less than that of the granules of the other neurosecretory cells, the small populations of granules in the axons makes the distinction difficult. The existance of the neurosecretory cells at the proximal chiasma, as well as the two distal groups, suggest that the optic lobe of the cockroach may have a neurosecretory system of similar complexity to that of the crustacean eye stalk.

The major factor which has prevented the earlier discovery of this system is the staining reactions of the NSM. The dependence upon general histological stains, such as the

azan technique and Mallory's triple stain, make the task of axon tracing almost impossible. This is further complicated by the small amount of NSM in the axon tracts.

The specialized contact zones between the two types of neurosecretory axons appear to represent true synapses in all morphological characteristics. Synapses between small granule axons (type B fibres) and large granule axons (type A fibres) may indicate the control of neurosecretory axons by aminergic axons. Similar types of synapses have been reported from other insect species and probably represent true synapses, whereas other "synaptoid" junctions probably represent release sites for NSM (see Scharrer, 1968 for discussion). These synapses could be the control site for either the regulation of synthesis of NSM in the perikaryon, or the transport of NSM down the axons, or the release of NSM from the axons, or a combination of all or any of these. The perikarya of the cells containing the small granules have not been found so it is impossible to say from where this group of neurosecretory cells is controlled.

The part of the neurosecretory cell at which synapses occur is not particularly clear in this case. Although the synapse region is fairly close to the perikaryon, no synapse could be found where the post-synaptic element could be traced back to the cell body. Adiyodi and Bern (1968) showed that the morphology of the neurosecretory cells in

the pars intercerebralis of Periplaneta was essentially the same as "conventional" neurons, possessing an axon which has dendritic and collateral branches. Synapses were assumed to occur on these branches. In the case of the optic lobe neurosecretory cells, the post-synaptic elements tend to be thinner than the axon proper. Also, the axons sometimes have branches upon which synapses occur. Although actual proof of the location of the synapses is lacking, there is circumstantial evidence for the existance of dendritic branching.

The presence of en passent release sites along the axon tracts raises important questions as to the target organ of the released NSM. The internal extracellular connective tissue of the optic lobe is essentially isolated from the haemolymph. This is contrary to the case of other neurohaemal organs where the stroma is in continuity with the haemolymph (see Scharrer and Weitzman, 1970). In these latter cases, the released NSM has direct access to the haemolymph and hence the target organ. The only tissue in the optic lobe which could be affected by the released NSM could be other neurons or glial tissue. If the nervous tissue is the target tissue, then the NSM may depress or activate or change the stimulus threshold of particular groups of neurons. This type of control would have far-reaching physiological implications. Of course, the other unknown factor is the location of the axon terminals of these neuro-

secretory cells. The axons appear to run into the brain, but beyond this their pathway is entirely unknown. Further exploration of the axon pathways and terminations would perhaps provide further clues on the function and possible target tissues of the NSM contained within the optic lobe neurosecretory cells.

These acidophilic cells in the optic lobe constitute a new group of neurosecretory cells in insects. At the moment their function can only be speculated upon, but from their location it would appear that they may be somehow connected with the eyes and respond to changes in the light intensity or photoperiod. The physiological clock controlling activity rhythms has been located in the optic lobes of Periplaneta (see review by Brady, 1969), and these cells may well be associated with this clock mechanism.

THE OPTIC LOBE NEUROSECRETORY SYSTEM AND CIRCADIAN RHYTHMS

INTRODUCTION

The driving oscillator for the circadian rhythm of locomotor activity in cockroaches appears to be situated in the optic lobes (Nishiitsutsuji-Uwo and Pittendrigh, 1968b). This is in contrast to the mechanism controlling circadian rhythms of locomotion proposed by Harker (see 1964 work for summary). The essence of Harker's study is that the rhythm of locomotion is controlled and endogenously timed by two pairs of neurosecretory cells in the sub-oesophageal ganglion; that the sub-oesophageal gangion must receive a supply of neurosecretory material from the corpora cardiaca by way of the nervus corporis allati II (NCA II) for the maintenance of the rhythm; that the rhythm has an ability to phase-shift related to a second clock which responds to light received by the ocelli. Subsequent work has failed to substantiate Harker's conclusions (Roberts,1965a,b,1966; Brady, 1967a,b; Nishiitsutsuji-Uwo and Pittendrigh, 1968a,b; Nishiitsutsuji-Uwo *et al.*, 1967). This later work , reviewed by Brady (1969), indicates that the suboesophageal ganglion does not contain the "clock" mechanism and that the corpora cardiaca and neurosecretory cells of the pars intercerebralis are not necessary for the expression of the rhythm and that the compound eyes and not the ocelli are the receptors through which entrainment of locomotor activity to cyclic light

regimes is achieved. The optic lobes have been implicated as the site of the oscillator and an intact neural pathway to the thoracic ganglia is necessary for the expression of the rhythm.

A previously unknown group ofneurosecretory cells has been found in the optic lobes of Periplaneta americana (Beattie, 1971b; also Chapter 2 of this thesis). The anatomical location of these neurosecretory cells indicates that they may be involved in the control of circadian locomotor activity. Both histological and surgical procedures have been used in an attempt to show any relationships between the rhythm of locomotor activity and the neurosecretory system in the optic lobe.

MATERIALS AND METHODS

Newly moulted adult animals of both sex were isolated from the main laboratory culture and kept in separate cages.

For the histological study, only males were used. They were kept in a light regime of 14 hours light and 10 hours dark (LD 14:10). Animals were sacrificed at 10.00 a.m., 2.00 p.m., 6.00 p.m., 10.00 p.m., 11.00 p.m., midnight, 1.00 a.m., 2.00 a.m. and 6.00 a.m. by injection of Bouin's fixative into the abdomen. The heads were cut off and the supra-oesophageal ganglia with attached optic lobes were dissected out and placed in fresh fixative. Frontal sections were stained with azan (Hubschman, 1962). The slides were arranged in randomized groups so that batch differences would not introduce

systematic errors. The major and minor axes of the whole cells and their nuclei were measured with a calibrated graticule at a magnification of 640X. At this magnification, the dimensions could be measured to an accuracy of 0.05 units or 0.8 μ. The area of an oval with the measured parameters as major and minor axes were used as an estimate of cell and nuclear size. Estimates of volume using only two parameters and assuming the third can lead to serious errors (see Brady, 1967c, for a discussion of this point), so the cross-sectional area was used. Only one series of animals (i.e. 1 animal at each time) has been analysed.

To expose the posterior face of the optic lobes in live animals, an incision was made along the posterior margin of the eye to about halfway down the side of the head capsule. Another cut was made from the median end of the first incision to the posterior margin of the head capsule. This allowed the posterior part of the head, which contains the main madibular muscles, to be pulled back to expose the optic lobe. All surgical procedures were carried out under carbon dioxide anaesthesia.

Thirteen males and 7 females were used in experiments to show the effect of removing the optic lobe neurosecretory (NS) cells. The followingprocedures were used: either the NS cells of each optic lobe were destroyed by thermo-cautery using an electrically heated needle, or one optic lobe was severed from the brain and the NS cells of the other optic lobe

cauterized, or one optic lobe was extirpated and the NS cells of the opposite optic lobe cauterized. Integumentary wounds were not sealed with wax as the blood clotted rapidly and there was little bleeding.

In another series of experiments, 9 pairs of animals were grafted parabiotically so that their blood was continuous through the pronota. One member of each pair had its optic lobes removed (the runner) and the other member of thepair had its optic lobes intact but the legs were cut off (the rider). Two controls were used. In one, there was no blood continuity between the pair, and in the second, both members of the parabiotic pair had their optic lobes removed.

Activity was measured in LD 12:12. The activity of the animals was recorded in running wheel actographs. These were essentially the same as used by Roberts (1960). The wheels were constructed from balsa wood and "Terylene" mesh. Each wheel was 15 cm diameter. The supporting axle passed through a 2.5 cm cube of balsa wood mounted centrally on one face of the wheel. Crossed pins were used as axle bearings, the shaft resting on one pair and held beneath the second, furthermost pair of pins. A circumferential slot was cut in the shaft sufficiently wide to accommodate one pin so that the shaft would not "run out" during rotation. Water and food were supplied through a central hole in the side of

the wheel opposite the axle mountings (similar to Roberts, 1960).

To measure the activity, a small cylindrical magnet with both poles at one end was mounted co-axially on the shaft of the running wheel in such a position that it activated a magnetic reed switch mounted on the bearing supports. This gave two events for each 360° rotation. The reed switch completed a circuit which activated a relay. The relay movement mechanism was modified so that a light-weight marker (plastic drinking straw) could be mounted on it. The marker made contact with a smoked drum which rotated once every 24 hours. Five activity event markers and a light regime marker were arranged so that they concurrently marked the drum. The light regime marker was coupled to a time switch controlling the lamps in each light-tight box.

Each light-tight box was about 30cm x 30cm x 30cm internal dimensions which was sufficient to accommodate two running wheels (the activity of one animal had no apparent effect upon the other animal in the same box). The lids of the boxes carried a 4W fluorescent lamp mounted on an aluminium strip immediately beneath a metal light-proof vent. The aluminium strip was carried beyond the edges of the vent on the outside to help dissipate heat generated by the fluorescent lamp. When the lamp was on, it raised the internal temperature 2°C above the external temperature (25° to 27°C). The inner surface of the light-proof vent was painted

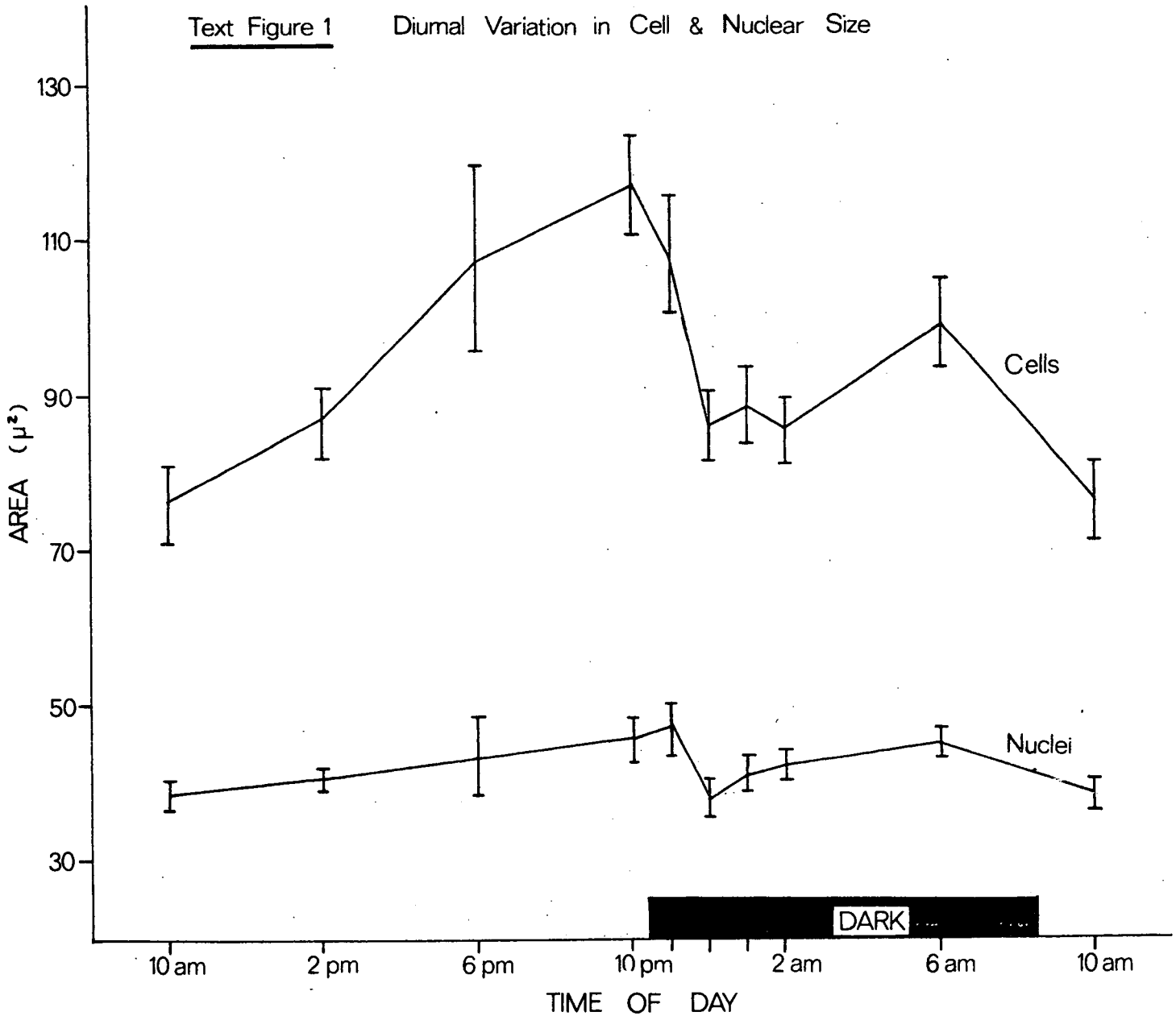
matt black during its construction to prevent light reflections. The edge of the lid was rebated and a flange added to the outer edge so that it projected down the sides of the box. A strip of foam rubber ran around the top edge of the box and around the rebate cavity to take up any unevenness between the lid and the box and to make a light tight seal. All joints were filled with opaque filler and both box and lid were painted matt black on all surfaces.

RESULTS

Circadian Variation in Cell and Nuclear Size

The variation in cross-sectional area of the NS cells and their nuclei is shown in Text figure 1. For cell size there is a highly significant variation for the different times of the day (see Tables I and II*; Text fig. 1). The calculated minimum significant difference for the means is $13.8/u^2$ (5% level of probability). From this value, 6.00 p.m., 10.00 p.m., and 11.00 p.m. are significantly larger than the mean size at 2.00 p.m. and midnight. Similarly, the cell area at 6.00 a.m. is significantly greater than the cell size at 2.00 a.m. and 10.00 a.m. Thus, there are two peaks in the size of the cells during 24 hours. The peak at the onset of darkness is well marked, but the peak at dawn is not so well shown. Perhaps this is because of the wider sampling times at this period. These peaks in cell size coincide fairly closely with the peaks of locomotor activity. The dusk period of activity is the major peak and the dawn peak

* Tables on pages 48 and 49.


Text Figure 1 Diurnal Variation in Cell & Nuclear Size
130
110
90
70
50
30
AREA (μ²)
Cells
Nuclei
DARK
10 am
2 pm
6 pm
10 pm
2 am
6 am
10 am
TIME OF DAY

is the secondary peak. Roberts (1960) indicated that the secondary peak depends on the lighting regime, and is the major peak in short dark period days (e.g. LD 23:1).

For variation in nuclear size, significant differences are found (see Tables I and II, Text fig. 1), and the significant peaks occur at dusk and dawn. The 11.00 p.m. peak is significantly different from the mean nuclear size at 2.00 p.m. and midnight. Likewise, the 6.00 a.m. peak is significantly greater than the midnight and 10.00 a.m. means.

The staining characteristics of the NS cells was variable and appeared to depend more on the staining batch rather than on the different times. Thus, at any particular time, the NS cells may be red or dark blue. This variation probably arises from the extent to which the azocarmine was differentiated. The timing of the differentiation step of the staining procedure appears to be quite critical.

The amounts of NS material in the cells was difficult to assess mainly because of the variable staining of the cytoplasm. However, some of the cells at 11.00 p.m. did not appear to contain very much stainable material and the cytoplasm appeared vacuolar. This vacuolization of the cytoplasm appeared to be the only sign of change in the amount of NS material in the cytoplasm.

Cautery of NS Cells

Of the 20 animals in which destruction of the NS cells was attempted, 6 returned to rhythmic locomotor behaviour, 3 showed doubtful signs of a rhythm before they died (3-28 days post-operative) and the remainder died without regaining a rhythm. Some of the animals which showed a return of rhythm were fixed in Bouin's fixative and the optic lobes sectioned. All showed at least some NS cells, indicating that cautery had been incomplete.

Parabiotic Pairs

Animals from which the optic lobes had been removed often showed intense activity during the period immediately following the operation but on subsequent days they remained almost inactive. When another animal was grafted onto the operated animal, the pair showed increased activity. However, this activity was arhythmic and was similar to the controls (i.e. a pair with no blood continuity and a pair both of which had the optic lobes extirpated).

DISCUSSION

The maximum size of NS cells and their nuclei show a correlation with locomotor activity, but this need not necessarily imply a causal relationship. The change is size of the cells may be a second order process and only follow changes directed from another source. Also, the results can only be taken as a preliminary investigation since the numbers of animals are too small. Only one animal for each time is

probably not sufficient to draw any concrete conclusions. Another criticism of the results is that there are no control measurements on other cell groups. As Brady (1967c) found, motor neurons show a fluctuation in size which tends to follow the same pattern as NS cells in the sub-oesophageal ganglion. A further factor which may influence the significance of the measurements is the magnification at which the sections were viewed. At 640X, an error of $\pm 0.8\mu$ is possible in measurements. This could lead to errors of up to 13% in whole cells and about 20% in nuclear measurements. With errors of this magnitude, the significance of the results must be regarded with care. A further influence on the results may arise from the subjective elements in measuring (i.e. estimating between graduation marks), and the suspicion that there may be a relationship between cell size and locomotor activity. The measurements may be read consistently higher or lower, quite unconsciously, knowing the time of day at which the animal was sacrificed.

Apart from the criticisms mentioned above, there is a good indication that a change in the size of the NS cells and their nuclei which is correlated with locomotor activity. The uncertainty of the present results could be reduced by further measurements paying care to possible sources of error.

In the experiments where destruction of the optic lobe NS cells was attempted, it was evident from the autopsies that a degree of uncertainty was introduced by the techniques

used. Thermo-cautery did not appear to be effective in destroying the NS cells in every case. Also, at the outset of this series of experiments, the full anatomical distribution of the NS cells was not realized. Initially it was thought that the NS cells were confined to a group on the dorsal aspect of the posterior face of the optic lobe. However, during later investigations, the NS cells were found to extend ventrally to form an almost bilobed arrangement. Even if the dorsal part of the group was destroyed, the ventral portion would remain intact.

It appeared from this series of experiments, as well as from other workers results, that it is easy to upset the locomotor rhythm without actually destroying the underlying "clock" mechanism. Also, from these experiments, it would be impossible to conclude conclusively that the oscillatory centre had been removed rather than masking its expression. Instead of attempting to destroy the rhythm, as in the cautery experiment, the series of parabiotic grafts should show whether or not a rhythm could be induced by some factor transported in the haemolymph.

Removal of both optic lobes makes the animals very inactive within 24 hours. The initial post-operative activity is most probably due to shock induced by surgery. When an operated animal is loaded with the weight of another animal, irrespective of blood continuity between them, the level of activity is raised but does not show any rhythm. The loading

effect probably induces a stress situation and any movement of the rider starts a bout of activity in the runner. A similar bout of activity can be induced in an animal which has had its optic lobes removed by mechanically disturbing it. From this series of experiments there is no apparent induction of a rhythm in the lobeless runner by the intact rider. Of course, the mechanical stimulation from the rider may mask out any rhythm present. Alternatively, the interchange of haemolymph between the two animals may be minimal and insufficient to induce a rhythm in the runner.

According to the conclusions of Harker (1964), removal of the optic lobes should not upset the rhythm since the suboesophageal ganglion, the NCA II and the ocelli are still intact. However, care should be taken with this interpretation since the expression of the rhythm may be upset by the operation rather than removal of the "clock".

In view of Nishiitsutsuji-Uwo and Pittendrigh's (1968b) work, the present investigation does not give any further information on the location of type of oscillator responsible for the circadian rhythm of locomotion.

It may be significant that Jacklet and Geronimo (1971) showed the circadian rhythm in the frequency of compound action potentials from the isolated eye of Aplysia is dependent upon a certain population size of neurons. It is the interaction between the neurons which give rise to the circadian rhythm, and once the population size drops below

a critical value, the period of the rhythm of discharge becomes ultradian (ca. 7.5 hours).

From a structural point of view, the optic lobe NS cells may be a second order system since they receive synapses from some unknown neurons (see Chapter 2 in this thesis). If the NS cells are involved in the expression of locomotor activity, then they may change the activity of neurons which control locomotor activity. Such neurons (inhibitory) are present in the corpora pendunculata (e.g. Wigglesworth, 1965). The path by which NS material could reach these neurons may be entirely within the nervous system since probable release sites of NS material were found within the optic lobe (see Chapter 2). This would be a novel mechanism for the NS material to reach its target tissue since transport through the haemolymph would not be employed. The active factor would reach its target by extracellular pathways entirely within the nervous system.

Table I Diurnal variation in whole cell and nuclear area.

TIME	WHOLE CELLS			NUCLEI	
	n*	$\bar{x}$*	$s_{\bar{x}}$*	$\bar{x}$*	$s_{\bar{x}}$*
10.00 a.m.	61	76.2	2.6	38.6	1.02
2.00 p.m.	64	86.9	2.5	40.5	0.85
6.00 p.m.	20	107.5	6.0	43.6	2.54
10.00 p.m.	50	116.8	3.3	45.6	1.50
11.00 p.m.	58	108.1	3.8	47.0	1.65
Midnight	58	86.0	2.3	38.2	1.25
1.00 a.m.	52	88.5	2.6	41.6	1.34
2.00 a.m.	59	85.5	2.3	42.7	1.16
6.00 a.m.	72	99.3	2.8	45.5	0.99

*n - number of cells measured; $\bar{x}$ = mean area (μ^2);

$s_{\bar{x}}$ = standard error of mean.

Table II Analysis of variance of cell and nuclear area for different times of day.

CELLS

Source of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance Ratio
Between classes	75,129	8	9,391	19.65****
Within classes	231,687	485	478	
Total	306,816	493		

**** = Significant at 0.1% level of probability

NUCLEI

Source of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance Ratio
Between classes	6,753	8	844	9.59****
Within classes	42,880	485	88	
Total	49,633	493		

**** = Significant at 0.1% level of probability

SEGMENTAL BLOOD VESSELS

INTRODUCTION

Segmental blood vessels (SBV's) are accessory heart structures which allow the distribution of blood to the lateral areas of the body. Their occurrence was first reported by Alexandrowicz (1926) when he described four pairs of vessels in the abdominal region of Periplaneta (Blatta?) orientalis. The first pair are situated between the second and third abdominal segments and the remaining three pairs between each of the successive posterior segments. Each vessel arises ventro-laterally from the heart and runs laterally to end amongst fat body cells at the lateral boundary of the dorsal diaphragm (McIndoo, 1939). However, Nutting (1951) states that the vessels divide distally and a branch passes to either side of the tergo-sternal muscle. McIndoo (1939) also found short vessels in the meso- and metathoracic segments.

McIndoo (1939) studied these vessels histologically and came to the conclusion that the walls contain small amounts of nonstriated muscle. However, Nutting (1951) states that the walls are non-muscular. The mass of cells which form the valvular apparatus were found to be multinucleate (up to 15 nuclei) and the cytoplasm of these cells had a spongy or fibrous appearance.

The innervation of the vessels was described initially

by Alexandrowicz (1926). A branch of the lateral cardiac nerve passes ventral to the vessel while the main portion passes dorsally. From the ventral branch numerous small nerves run, to, and terminate in, the mass of cells forming the valve. These fibres are very much entangled and varicose. Laterally, the vessels are innervated by parallel tracts of fibres which run down its length. These fibres originate from the lateral cardiac nerve. McIndoo (1939) has shown that axons from some of the tripolar ganglion cells of the lateral cardiac nerve innervate the segmental blood vessels.

Functionally, the SBV's act in an arterial capacity (McIndoo, 1939). The valve at the neck of the vessel allows movement of blood away from the heart only. In isolated preparations of the heart, liquid was shown to circulate through the vessel and return to the heart via the incurrent ostia.

The present study is a reinvestigation of the structure of the segmental blood vessels at the ultrastructural level, but vitally stained whole mounts of the vessels has been used to show particular aspects of these structures.

MATERIALS AND METHODS

Adults of both sexes and larvae of the cockroach, Periplaneta americana were used throughout this study. The insects were taken from a laboratory culture maintained at 25°C and 70% relative humidity.

Two methods were used for vitally staining the nervous tissue of the SBV's. The reduced methylene blue technique (Pantin, 1946) was used for permanent whole mounts; the fixed tissue being mounted in polyvinyl alcohol. A vital staining technique for differentiating neurosecretory axons (Beattie, 1971a, also chapter in this thesis) was employed to trace this type of nervous tissue.

For electron microscopy, the isolated dorsal surface of the abdomen was flooded with ice-cold 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) containing 0.4M sucrose. The overlying alary muscles were removed and the vessels attached to a piece of heart dissected out and placed in fresh fixative. The tissue was fixed for one hour at $0^{o}C$, before being washed in buffer for a further one hour. Post-fixation was achieved with 1 or 2% OsO_4 in the same buffer. The tissue was dehydrated in a graded alcohol series, cleared in propylene oxide and embedded in Epon 812. Some tissue was block stained during dehydration by incorporating 1% phosphotungstic acid in the 70% alcohol and extending this step of the dehydration to one hour. Silver sections were cut on an LKB Ultratome II using a diamond knife. Sections were stained with uranyl acetate and lead citrate and viewed with an AEI EM6 electron microscope.

RESULTS

Methylene Blue Technique

Vital staining with reduced methylene blue gave

results which were quite consistent with those of Alexandrowicz (1926) and McIndoo (1939) (Fig. 1). However, in these two papers no reference was made to the termination of the parallel fibre tracts which run down the SBV's. In good preparations, where the whole of the SBV can be dissected out and where the staining is sufficiently good, these axons are seen to terminate before the end of the SBV (Fig. 1). The terminations show no specialization but the axon merely becomes thinner and eventually stops. During the courses of these fibre tracts, groups of axons or single axons divide off from the main group and form another parallel tract (Fig. 2a). This may be the branching of a single axon in some cases, but this type of preparation does not allow such a decision to be made. All axons in the distal part of the SBV appear to be identical when stained by this technique in that they all have a varicose appearance.

Acridine Orange Technique

With this technique, all the fibre tracts running down the SBV appear as bright orange-red fluorescing tracts when viewed with blue light (Fig. 2b). They have a varicose appearance and show branching in the same manner as with methylene blue staining. This orange-red fluorescence reaction is indicative of neurosecretory material (Beattie, 1971a). The axons which terminate within the valve of the SBV's also give a positive reaction for neurosecretory material.

In the walls of the SBV's numerous ovoid green-fluorescing nuclei are to be found. More often than not, they lie in rows with their long axes oriented parallel to the length of the SBV.

Electron Microscopy

The extreme thinness and delicacy of the SBV wall does not allow a full evaluation of cellular structure as revealed at the light microscope level of resolution and magnification. However, at the ultrastructural level of investigation, the SBV has shown itself to more complex than previously thought. For the sake of expediency, the results will be presented in two parts. First, the structure of the SBV wall will be dealt with and secondly, the structure of the valve of the SBV.

The Wall of the Segmental Blood Vessels

The wall of the SBV is composed of three elements; longitudinal strands of extracellular collagenous material set in a basement lamella, cells which are most probably fibroblasts, and nerve axons. These three components form a wall which is 1-2 μm thick.

The individual collagen strands vary in diameter from about 0.25 μm to about 1.0 μm, but the majority are less than 0.5 μm. They are spaced at intervals of less than 1.0 μm around the inside and outside surface of the SBV (Fig. 3). Each strand is composed of tightly packed collagen fibres which, when seen in longitudinal section, show their characteristic periodic banding of about 55nm. All

the collagen fibres are oriented approximately parallel to each other within a particular strand (Fig. 4).

The strands take on varying electron-densities depending on the staining technique used. When the tissue is stained after sectioning, the larger strands are electron-translucent and in few cases show any detail in the centre of the strand. Only the periphery shows more electron-dense fibrous structures. However, when phosphotungstic acid is used as a block stain, the whole strand is strongly stained.

For most of the length of the SBV, the strands show little evidence of branching and gently fan out towards the lateral end. At the proximal end of the SBV they bunch together (Fig. 5a) and probably join up with the connective tissue associated with the heart wall (Fig. 5b) and the dorsal diaphragm. These latter strands join the heart on its ventral surface. The distal attachments of the SBV's were not studied in detail because in this region they fan out and become covered with fat body tissue. However, several cases were examined and the strands of connective tissue were seen to become incorporated into the stroma surrounding the fat body cells on one side of the vessel lumen only. The other side of the vessel is formed by fat body cells only (Fig. 6). Also in this region, there is no cellular component in the wall as there is in the more proximal portions. The actual lateral attachment of the

connective tissue strands to the body wall or internal organs was not investigated.

The basement lamella or stroma forms at least one layer around the external surface of all cellular components as well as the collagenous strands. It follows the general profile of the cellular surface and does not extend into "tight corners" of folded plasma membrane. It is also excluded from narrow intercellular spaces. The stroma is composed of flocculant or fibrous material (Fig. 4) which can form a laminated layer up to 2 µm thick (Fig. 8). The laminations are merely different degrees of aggregation of this material and perhaps each lamina is lain down at different times. Usually, the stroma only forms one lamina around the other wall components.

The fibroblasts form a discontinuous layer between the connective tissue strands but sometimes there are desmosomes between adjacent cells (Fig. 3, 7a). Fine cytoplasmic extensions protrude towards and between adjacent strands and on occasions almost surround them. These cells can also encircle individual axons or bundles of axons (Fig. 7b,c). In these areas, they seem to fulfil the role of glial cells, but cytoplasmic extensions can be seen arising from nearby parts of the cytoplasm which are associated with the connective tissue strands. In the proximal region of the SBV, axon bundles have well formed

glial sheaths and appear to be quite distinct from the surrounding fibroblasts (Figs. 8, 9a). The dimensions of any particular fibroblast could not be determined because of the cytoplasmic ramifications. The distance between adjacent nuclei is at least 20 /um, so one cell would be at least this size.

Fibroblast nuclei are usually flattened and ovoid to be accommodated within the SBV wall (Fig. 3). However, towards the proximal end of the vessel, they are irregular in form (Fig. 8). They measure about 6 /um in their longest axis. The nuclear membrane may be smooth or highly infolded (Fig. 9b). The chromatin is usually peripheral and the remainder of the nucleus has numerous small dense granules scattered throughout.

In the cytoplasmic ramifications as well as in the perinuclear area, elements of rough endoplasmic reticulum (RER) are found. The material in the cisternae has medium electron-density and has a fine granular appearance. The cisternae are dialated in places (Figs. 5a, 10a, 12a). In these regions, there are few ribosomes attached to the membranes. In a few very large dilations, the contained material has a fibrous appearance (Fig. 5a). Ribosomes also occur singly or in clusters throughout the cytoplasm (Fig. 7a) as well as attached to the outer nuclear membrane (Fig. 7b, 9b).

Mitochondria are usually small and dense with few

cristae. They are spherical or elongate and rarely exceed 0.5 /um in length. The majority are about 0.3 /um in diameter (Figs. 10a, 10b, 12b). Intramitochrondrial dense granules are found in only a few cases.

Golgi bodies are fairly frequent and occur in any part of the cytoplasm (Figs. 10a,b). They are generally small (less than 1 /um) and are composed of 3-4 cisternae. The material within the cisternae is electron-translucent. A cloud of vesicles (50-60nm in diameter) are associated with the Golgi bodies. The vesicles contain a peripheral layer of election-translucent material while the centres appear to be clear.

Microtubules are common and are present throughout the cytoplasm (Fig. 5a). They occur singly or in small groups. Mainly, they are oriented parallel to the long axis of the vessel. Each microtubule is surrounded by a halo of electron-transparent material. This halo may have resulted from leaching during tissue preparation or by the presence of an "unstainable" substance.

In places, the plasma membrane forms a bulge filled with irregular shaped vesicles which vary in size from 60nm to over 250nm (Figs. 3, 7a, 10a, 10b, 11b). These vesicles are almost electron-transparent. The bulges sometimes have a neck and the whole structure is almost pinched off from the cell. In some cases, the bag of vesicles appears to be separate from the cell, but since

they are quite close to the cell plasmalemma, the possibility that they are still attached by a narrow neck cannot be discounted. A bag of vesicles is never found at any great distance from the plasma membrane, so it is unlikely that they have an independent existence. Rarely do these bags of vesicles show any consistent relationship with any other cell organelle. In one case, there appears to be a continuity with dialations of the ER, but this may be a fortuitous occurrence (Fig. 12a). The fate of these bags of vesicles is equally unclear. In some examples, it is difficult to delimit the surrounding plasma membrane, and in others, clear areas are adjacent the plasma membrane (Fig. 12b). These latter areas have a discontinuous, indistinct membrane encircling it. These observations suggest that the plasma membrane and the vesicles break down and leave a clear area (Fig. 11a).

The dorsal diaphragm (Fig. 13a) has essentially the same structure as the SBV wall. It possesses strands of collagenous material embedded in a basement lamella. The fibroblasts have dilated cisternae of RER filled with amorphous medium-dense material. Also, the fibroblasts do not ramify through the dorsal diaphragm to the extent to which fibroblasts invade the wall of the SBV.

All axons in the SBV's are of the neurosecretory type. This confirms the observations made on whole mounts using acridine orange vital staining. In the proximal

region of the SBV's, the axons tend to be grouped into bundles (Figs. 9a, 13b) while in the more distal region the axons tend to separate and groups of 1-3 axons are more usual (Figs. 7b, 12b). There is a fairly well developed glial layer encircling the axon bundles in the proximal region (Fig. 9a) and often individual axons are isolated by glia (Fig. 7c). However, more distally, the glial layer is not so well developed (Fig. 3) and it becomes difficult to distinguish glial tissue from fibroblasts. Occasionally, cells which partially enclose axons are found to have fibroblastic characters in other parts of these cells (Fig. 7b). On ultrastructural evidence, the difference between glial cells and fibroblasts is not great, but this does not preclude a physiological difference.

Concurrent with this change in the glial investment is the occurrence of more axons which have little or no cellular layer between them and the haemolymph, and the reduction in number of axons in any particular bundle. Quite often, the axons have a small diameter and occur singly (Fig. 14a). This, together with the fact that no axons or fibroblasts are present in the most distal portion of the vessel (Fig. 6), indicate that the axons taper off and terminate in the wall of the SBV. Also, counts of the numbers of axons at different levels along the SBV indicate that there is only a small increase in numbers in the distal direction. The increase in numbers of axons may be counteracted to some

degree by terminations at various positions down the SBV and thus mask a proportion of any increase due to axonal branching. It would appear then, that axons run almost the whole length of the vessel and terminate in the distal portion.

The neurosecretory axons can be classified into three types on their ultrastructural appearance. The most common type is characterized by electron-dense elementary granules which range in diameter from 100 to 200nm, but with the majority in the region of 160-170nm (e.g. Fig. 14b). The second type possesses elementary granules which have a medium electron density and fine granular contents (e.g. Fig. 15). The electron density of these granules is variable. Some are almost devoid of stainable contents while others have well packed granular contents. The diameter of these electron-translucent neurosecretory granules is usually greater than that of the electron-dense type and some are more than 300nm across. In some axons, the majority of elementary granules are electron dense, but there are also a few electron-translucent granules present. Similarly, there are cases where the reverse situation is true, i.e. the majority are electron-translucent but with a few electron-dense granules. However, there does not appear to be a continuum from one type to the other, and the two types are readily distinguishable. The rarest type of neurosecretory axon contains small electron-dense

granules (Fig. 16). These are 100-120nm in diameter. Quite often, many of these granules are ellipsoid.

The three types of neurosecretory axons are not separated into different bundles, but occur mixed together. Since the large electron-dense granule type is the most common, many bundles contain this type exclusively. The other two types are present in the axon bundles of the proximal portion of the SBV and are rarely found in the extremities of the vessels.

The axoplasm of all three types of neurosecretory axons contain microtubules, elements of endoplasmic reticulum and small mitochondria. The large electron-dense granule type of axon also have small dense granules (20-40nm diameter) scattered between the elementary granules (Fig. 9a, 14b, 15, 16). These small granules do not appear to have a limiting membrane and could perhaps be glycogen.

The neurosecretory axons are usually wrapped in glial tissue in the proximal part of the SBV, but a short way down the vessel many axons do not have a glial sheath. In these naked areas, the axons only have the basement lamella separating it from the haemolymph. The axonal plasma membrane is usually irregular in the naked areas, unlike its smooth appearance when it is adjacent glial cells or other axons. These naked areas are variously modified for the release of neurosecretory material and show minor differences between the three axon types. These modifications

are very evident in animals killed and fixed during their period of greatest locomotor activity (about 1 hour after the onset of darkness). Animals killed during the daylight hours do not show any evidence of NSM release.

The axons containing large dense granules possess a cluster of vesicles adjacent to the plasma membrane in the release areas (Figs. 12b, 17a). The contents of the vesicles are quite variable, many having dense contents while a fewer number have medium or clear contents. The diameter of these vesicles is in the region of 70nm. The axons with electron-translucent granules show a similar aggregation of small vesicles at release sites (Fig. 17b). In this case, the contents of the vesicles are much less dense than in the former axon type but the diameter are of similar magnitude. The axons which have the small electron-dense granules also show evidence of release (Fig. 16), although the release sites are not always directed towards the haemolymph. They may occur inside the bundle of axons and be adjacent glial tissue. In either locality the form of the release site is similar. A cloud of small vesicles (30-50nm diameter) is adjacent the plasma membrane. The vesicles usually have clear contents. The axoplasm immediately inside the plasma membrane may be electron dense and appears to form the focal point of the release site.

Evidence of exocytosis of neurosecretory granules

has been found on only a few occasions (Fig. 7b, 14b, 17c). All of these examples have been found in the axons containing large dense granules, but this may be a reflection of the greater number of these axons only and not an exclusive property of this axon type. Exocytosis has been found where the axon abuts other cellular components quite near the blood space, and sometimes towards the haemolymph. A sphere of electron-dense material, without a limiting membrane, is situated within a cavity in the axonal plasma membrane (an omega figure). The size and density of this material is quite similar to that of a neurosecretory granule. There is also evidence for the formation of small vesicles from an omega figure (Fig. 17c). The reason why exocytosis should be found in these intercellular spaces is not entirely clear. It would be expected that exocytosis would be confined to naked areas of the axons as this would be the quickest way for NSM to reach the circulating haemolymph. Perhaps it is the confining effects of the intercellular space that allows the exocytosis process to be visualized. The NSM would take longer to diffuse away from the site of release under these restraining confines, whereas on the naked aspect of the axon, the NSM is rapidly lost in the surrounding haemolymph. This idea gains support from the observation that the plasma membrane of all types of naked axons is often scolloped and the size

of these indentations is of sufficient magnitude to have been derived from a neurosecretory granule (Figs. 16, 17b). This scolloping effect is often evident where there are small vesicles present in the adjacent axoplasm, but does not exclude cases where it is present and there are no small vesicles.

Another modification of the naked axon membrane is shown by irregular infoldings to produce small projections. These appear to be produced at the expense of the axon, consuming portions of the axoplasm, and leaving the basement lamella quite smooth over the infolded area (Fig. 14b). The method by which this increase in surface area of the axon is achieved and the reason for such an increase is not clear. This type of axonal membrane modification is not a particularly common occurrence, but since it is only associated with naked axons, it could have some involvement with the release of NSM.

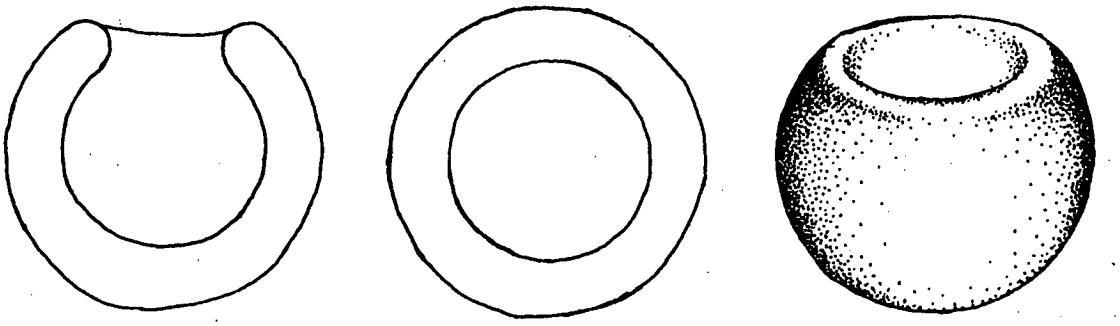
Coated vesicles and coated caveolae are found in some neurosecretory axons (Figs. 13b, 15, 18a). They are more common by far in animals which have been killed and fixed during the early part of the night, and rare in animals processed during the daylight hours. Usually, one or two axons in a bundle possess coated caveolae while there is no evidence of them in the rest of the axons of the bundle. These structures appear to be limited to two types of neurosecretory axons; those with large dense granules and those with electron-

translucent granules. In both types, there does not appear to be any size difference in the coated vesicles or coated caveolae. Usually, the vesicle (or caveola) is about 60nm across and the coat is 15-18nm thick. The coat appears to be made up of fine spines projecting from the membrane of the vesicle. In some cases, the distal end of the spines appear to divide and each arm joins with that of its neighbour (Fig. 18a). This gives the effect of small vesicles packed around the main vesicle. The contents of the coated vesicles and coated caveolae are almost electron-transparent, but sometimes a fuzzy layer of material lies adjacent to the membrane.

The distribution of caveolae on the axon plasma membrane is not confined to the naked areas, but may occur in regions where there is glial tissue opposed to the axon. Also, in axons which have caveolae, the number of coated vesicles is remakably small. This disparity is difficult to reconcile, unless the coated vesicles lose their coats and resemble "plain" vesicles. However, there does not appear to be many such vesicles in the axoplasm near the active zones. An alternative explanation may be that the coated vesicles, once formed, are rapidly transported away from their site of formation by axoplasmic streaming.

Scyphosomes (or cup-shaped bodies) are found fairly consistently in the vicinity of release sites and more rarely in other parts of the axoplasm (Figs. 12b, 14a, 17a,

19a, 19b). The scyphosomes take the form of an invaginated sphere so that the single membrane forms both the inner and outer wall. When viewed from the side, they appear U-shaped with the opposite (or nearer) wall faintly shown. When seen from an axial direction they appear as an annulus.



Sectional Side View | Sectional Axial View | Three Dimensional Reconstruction

Structure of scyphosomes

The contents, bounded by the singly membrane, may or may not be electron dense. The enclosed space of the scyphosome does not show any dense contents. The diameter of the scyphosomes is approximately 100nm in any direction, so that the overall profile is spherical. The mode of formation of these bodies is not clear-cut, but they may be formed from the coalescence of small vesicles. There are irregular-shaped vesicles which could have been formed from the fusion of several small spherical vesicles present in the axons. Also, forms intermediate between these irregular

vesicles and scyphosomes are present (Figs. 17a, 17b). This may be a sequence of formation of the scyphosomes, the final shape being the most stable.

Comparison with the Neurosecretory Axons of the LCN

The three types of neurosecretory axons found in the SBV wall have counterparts in the LCN (Figs. 18b, 19a, b,c). This is only to be expected, since all the neurosecretory axons of the vessel are derived from the LCN. The types of release sites are also the same in both structures (Figs. 18b, 19a,b,c). The release sites in the LCN, although common during the day, are more numerous in animals sacrificed at night. Again, this corresponds with the situation in the SBV neurosecretory axons. The occurrence of coated vesicles and coated caveolae in SBV axons has its parallel in axons of the LCN (Fig. 19d). Likewise, scyphosomes are common to both locations (Figs. 19a, b).

Nerve Cell Bodies in the LCN

The cell bodies in the LCN appear to be of the motor or sensory nerve type, and there is no evidence for the production of neurosecretory granules (Fig. 20). Although the cytoplasm has all the features of a synthetically active cell (as in most nerve cells), there are no accumulations of elementary granules. The endoplasmic reticulum is extensive, and numerous Golgi bodies are scattered throughout the cytoplasm. There are a few dense granules associated with the Golgi bodies, some of which are 80-100nm in diameter

while others are large and irregular. These latter dense bodies are most probably lysosomes.

In one particular batch of sections, both cell bodies of one segment were present. Their ultrastructural appearance was identical, so it seems probable that all neurosecretory axons found in the LCN and SBV's originate from the ventral nerve cord and the corpora cardiaca. The ventral nerve cord probably contributed most of the axons since a relatively large nerve joins the LCN in each segment of the body, whereas the nerves from the corpora cardiaca are small in comparison.

Valves of the Segmental Blood Vessels

In fixed tissue, the valves appear to form a mass of ovoid cells occupying the mouth of the SBV's. There does not appear to be any opening between these cells. However, in living isolated preparations of the heart, the valves can be seen to open and close. The frequency of operation of the valve is the same as that of the heart. In many preparations, the valve does not always open during systole, but remains firmly closed. This condition may last for many heart beats. The valve then partially opens during one heart contraction and open and close fully for the next and subsequent few heart beats. The valve only partially opens during the next contraction and remains fully closed during following cardiac cycles. The number

of beats during which the valve is closed is usually approximately equal to the number of beats for which it is open; for example, the valve may operate for 6 or 7 beats and remain closed for the next 6 or 7 beats. However, in some preparations the proportion may be 12 to 3. This rhythm of valve operation remains constant over considerable lengths of time (up to 45 minutes) and has not been observed to change significantly for any particular valve during the survival of preparation. Also the rhythm is not the same for valves of different segements of the heart; one may cycle at 6:6 while the adjacent valve may cycle at 5:8.

A partial explanation of this function can be gained from the structure of the valve. The cells forming the valve are a rather odd type of muscle cell which is well innervated by granule-containing axons from the lateral cardiac nerve (Figs. 21, 22). The muscular nature of these cells was not recognised by previous workers (e.g. McIndoo, 1939).

The muscle cells have one side greatly infolded and interdigitated with adjacent muscle cells (Figs. 21, 22), while the rest of the cell membrane is relatively smooth and does not come into close contact with neighbouring valve cells (Fig. 8). In the interdigitated regions, nerve axons and their associated glial cells as well as small tracheae occur (Fig. 21,22). A thin basement lamella covers all

plasma membranes of the muscle cells except where very closely opposed membranes occur (Fig. 23). Occasionally, strands of connective tissue containing collagen fibres runs between the muscle cells (Fig. 22).

The myofilaments do not fill the major portion of the cytoplasm as in usual muscle cells, but are more or less restricted to the region of the cell where the plasma membrane is infolded (Figs. 22, 24a). Usually, the most peripheral myofibrils lie immediately beneath the plasma membrane. The myofibrils may consist of a few myofilaments or a group of myofilaments up to ca. 2 /um in diameter. The myofibrils do not form an organized absolutely parallel array, and within any one cell, may be oriented in any direction (Fig. 24a). Quite often, adjacent myofibrils are at right angles to one another. The thick and thin myofilaments within a myofibril are not well organized and a thick filament may be surrounded by numerous (up to 10 or 12) thin filaments. All myofibrils have prominent Z bands which usually form an irregular line (Figs. 21, 24a). Z bands are spaced at intervals of 2-3 /um. In favourable longitudinal sections of the myofibrils, A bands and I bands can be differentiated. There are also indications of a broad ill-defined H band.

Occasionally, interfibrillar junctions between the muscle cells of the valve and those of the heart wall are found (Fig. 24b). In these junctions, a dense layer immediately inside the plasma membrane of each cell lie

opposite one another. The thin myofilaments are inserted into this dense layer. The plasma membranes of the cells are parallel to each other and a thin layer of dense material occurs midway between the cells in the intercellular gap. The intercellular gap is ca. 30/um across. The dense layer into which the thin myofilaments are inserted occurs in a zone which would usually be occupied by a Z band. Because of the discontinuous nature of the valve cell myofibrils, the interfibrillar junctions are also discontinuous. There does not appear to be any modification of the plasma membranes adjacent these junctions (such as tight junctions or septate desmosomes). A similar modification of the cell periphery is shown when there are connective tissue strands adjacent the muscle (Fig. 25). Of course, the intercellular modification is absent in this case. The connective tissue abutting the muscle cell shows no sign of modification, so it would be premature to assume the existence of any specialized attachment between the muscle cells and the connective tissue strands.

A transverse tubular system is not well developed in these valve cells and occurs irregularly between the myofibrils (Fig. 21). However, the sarcoplasmic reticulum (SR) is quite well developed (Figs. 21, 24a, 26a, b) and forms diads with the transverse tubules. Cisternae of the SR surround most of the myofibrils on all sides. The SR sometimes penetrates through the myofibrils in the region of the

junction of the I and A bands and subdivides the myofibril into smaller bundles of myofilaments in these regions (Fig. 24a). The SR is not confined to the immediate vicinity of the myofibrils. In areas where there is a cytoplasmic space between the myofibrils and the plasmalemma, a cisternal layer of SR is often formed immediately beneath the membrane (Fig. 26b). Also, the SR sometimes occurs in close association with the plasmalemma abutting nerve endings (Figs. 26a, b). Continuities between the SR and annulate lamellae (Fig. 27) as well as between the SR and centriole complexes (Figs. 28a, b) are also present. In the latter two cases, a few ribosomes are attached to the membranes of the SR. This raises a question of terminology; what is the dividing line between sarcoplasmic reticulum and endoplasmic reticulum?

Mitochondria do not form a regular association with the myofibrils, although there is a tendency to form groups in the same region as myofibrils (Figs. 8, 22). The mitochondria are either nearly spherical being 200 to 400nm in diameter, or elongate (1-2 μm along the major axis). Sometimes, the elongate forms are curved or U-shaped. The cristae run transversely, and often intramitochondrial dense granules are present.

The nuclei are up to 7 μm in their greatest dimension and occupy only a small part of the cell. Most cells appear to have at least two nuclei, but in some of these

cases the same effect may be produced by a section through a single lobate nucleus. Chromatin forms a peripheral layer around the nuclear membrane as well as patches throughout the nucleus itself. Nucleoli are present and measure slightly more than 1/um across (Fig. 8). Short sections of the outer nuclear membrane are studded with ribosomes.

The greatest portion of the cytoplasm is devoid of organelles and is uniformly filled with granular material which is most probably glycogen. Clusters of ribosomes are found throughout the mitochondrial fields, in the perinuclear region, and in the infolded portion of the cell. Rarely, ribosomes are found to be attached to short sections of endoplasmic (sarcoplasmic?) reticulum.

Throughout the cytoplasm, a variety of other cell organelles are present in small numbers. Golgi bodies, about 1/um across, are present either close to groups of lysosomes, or near centriole complexes (Fig. 28b), or in relative isolation from other cytoplasmic structures. The clusters of lysosomes (Fig. 21) are found in any part of the cytoplasm. Annulate lamellae are also present in these muscle cells (Fig. 27). Up to 26 lamellae have been found in one stack, and the lamellae may be up to 2/um long. The material within the lamellae membranes has a fine granular appearance and short fibres extend outward from the annuli. The membranes of the lamellae sometimes extend laterally into the surrounding cytoplasm or loop back and form a continuity

between adjacent lamellae. Of those lamellae which extend into the cytoplasm, some are continuous with rough endoplasmic reticulum while others are continuous with elements of the sarcoplasmic reticulum.

Centrioles are occasionally found in clusters of up to 6 pairs (Figs. 21, 28a, b). In favourable sections, the nine triplet arrangement of tubules can be distinguished. (Fig. 28a). Spoke-like structures extend out perpendicular to the triplets to a rim of more electron-dense material. They arise opposite the central member of the triplet and make an angle of ca. 30^{o} to a radial line of the centriole. These spoke-like structures are not present throughout the whole length of the centriole, but there appears to be more than one series of spokes. Some of the centrioles appear to be partially enclosed at one end by a dense double-membraned structure (Figs. 28a, b). Ramifying throughout the centriole complex is a tubular system of endoplasmic reticulum, some regions of which have attached ribosomes. There are indications that this endoplasmic reticulum is continuous with the sarcoplasmic reticulum (Fig. 28a).

Vacuolar structures occur scattered through the cytoplasm (Figs. 26a, 27). These are of two types. First, spherical vacuoles with membranous and vesicular contents, and secondly, irregularly shaped vacuoles which usually have electron-lucent contents or more rarely, granular contents.

The muscle cells of the valve are innervated by

granule-containing axons. These axons are derived from the lateral cardiac nerve and penetrate into the valve by way of the SBV wall (Fig. 8). At this point, the axons are in groups of up to about 6 members and are invested with layers of glial tissue. There are few granules in the axons in this zone, but groups of neurotubules occupy a large part of the axoplasm. The axon bundles penetrate the infolded, interdigitated regions of the muscle cells (Figs. 21, 23). Here, the glial investment is reduced and the bundles tend to subdivide. There is a large increase in the number of granules in the axons in this region and they almost fill the available axoplasm. Axons finally become separated from each other and become continuous with the muscle cells to form neuromuscular synapses (Figs. 21, 22, 23, 24a, 26a, 26b, 29a). The synaptic cleft is 30-35nm across. Synaptic vesicles are variable in shape, some being spherical and others ellipsoid. There are a few larger spherical vesicles up to 70nm diameter. The synaptic zones usually have a few small mitochondria present. In the synaptic region, the axon may have one side adjacent the muscle cell while the remaining surface has glial tissue opposing it, or the axon may penetrate the muscle cell completely and there is no glial tissue present (e.g. Fig. 23). In some synaptic clefts, a layer of dense material is present on the post-synaptic membrane (Fig. 29a). This layer is about 10nm thick and has fine striations running across it at about

every 15nm. The neuromuscular synapses on the heart wall have similar structures (Fig. 29b). The muscle cells have numerous synapses (Fig. 22). It is perhaps worthwhile to mention again at this point, that most of the myofibrils are located near the infolded region of the cell. It is in this region where the neuromuscular synapses occur. With this structural arrangement, the synapses are as close as possible to the contractile elements of the valve cells.

Incurrent ostia. The incurrent ostia operate with each cardiac oscillation, closing during systole and opening during diastole. There is no independent control of this valve which contrasts with the operation of the excurrent valves in the SBV's. This difference in function of the two types of valve is reflected in their structure.

Each slit-like incurrent ostium is composed of a pair of inwardly directed muscular lips. The lips are one cell thick as is the heart wall. The overall electron density of the ostial lips is greater than that of the heart wall (Figs. 30a, 31b). The ultrastructure of the ostial lips and the heart wall is similar. The myofilament arrangement is of the visceral muscle type (one thick filament with 10-12 thin filaments surrounding it)(Fig. 30b), Z bands are prominent and the sarcomere length is approximately the same in both types of muscle (Fig. 30a). The sarcolemma is folded in both, and the infoldings are in register with the Z bands. Both have a transverse tubular

system, (Figs. 30a, 31a) but this does not have a regular arrangement. Diads (Figs. 30a, 31a) are formed with the sarcoplasmic reticulum which runs parallel to the myofilaments. Mitochondria are abundant in the peripheral projections formed by the folded sarcolemma (Fig. 30a). There are more mitochondria packed into these spaces in the ostial lips than in the heart wall. These mitochondria-packed projections often have a rectangular profile in the ostial lips whereas in the heart wall they are more rounded.

The ostial muscle is attached to the heart wall by intercalated discs (Figs. 31a, b). These discs, or interfibrillar junctions, only have the fascia adherens type contacts and septate desmosomes or tight junctions have not been seen in these regions. The interfibrillar junctions have a zig-zag course which is typical for this type of intercellular attachment. There was no indication of innervation of the muscular lips of the ostia. However, the heart wall does ~~not~~ receive axons from the lateral cardiac nerve.

DISCUSSION

All excurrent ostia of many orthopteroid insects have a mass of cells forming a valve (Nutting, 1951). This is the case with Periplaneta, and perhaps all orthopteroids have similarly structured excurrent ostia. Also, Opozynska-Sembratowa (1936) showed in Carausius that there is a plexus of nerve endings at the ostia. Whether these are incurrent or excurrent ostia in Carausius is not known, but Nutting (1951) only found excurrent ostia in the thoracic region in the species he studied (not Carausius). Innervation of valves in other species of insects does not appear to have been reported.

The structure of the muscle cells comprising the SBV valve is quite different from any other type of muscle so far described in insects (or for that matter, any other animal). The spherical form of the cell, and the paucity and distribution of myofibrils are the most distinctive features. The structure of the myofibrils themselves, is similar to other visceral muscles, in that they have 10-12 thin myofilaments surrounding each thick one in the A band (e.g. Smith, Gupta and Smith, 1966; Odhiambo, 1970; Schaffer, Vanderberg and Rhodin, 1967; Baccetti and Biliardi, 1969a, 1969b; Anderson and Ellis, 1967). The orientation of the myofibrils is, however, not parallel. A similar situation is found in the gut muscles of Glossina (Rice, 1970), where

myofibrils can be oriented in either a circular or a longitudinal or a diagonal direction.

The poorly developed sarcolemmal invaginations to form the transverse tubular system is also a character of visceral muscle (Anderson and Ellis, 1967; Schaffer, Vanderberg and Rhodin, 1967; Odhiambo, 1970), but in some cases it is extensive and forms diads and triads with the sarcoplasmic reticulum (Anderson and Ellis, 1967; Rice, 1970; Baccetti and Biliardi, 1969a, 1969b). Similarly, the sarcoplasmic reticulum may show various degrees of development (see references above). It would appear as if the degree of development of the transverse tubular system and the SR varies with the anatomical position of the particular muscle and with its function. One fact which links all visceral muscle into a particular type is the lack of regular arrangement of the TTS and SR and the position of diads. This is very evident when the regular structure of skeletal muscle is considered (e.g. Hagopian, 1966; Hagopian and Spiro, 1967; Smith, D.S., 1968). The reduction in these systems could possibly be related to the speed of action of the muscle (Anderson and Ellis, 1967).

The haphazard distribution of mitochondria in the SBV valve muscles also indicate that this is a "slow" type of muscle. Fast acting muscles, such as the flight muscles, have a very regular arrangement of mitochondria and myofibrils

(e.g. Smith, D.S., 1968).

The combined presence of annulate lamellae, centrioles, Golgi bodies, rough endoplasmic reticulum, lysosomes and ribosomes in the valve muscle cells is a unique feature. Annulate lamellae are usually found in developing oocytes as well as other undifferentiated cells and some differentiated somatic tissue (see review by Kessel, 1968). Golgi bodies, RER and lysosomes are well known organelles of synthetically active cells, while centrioles have a role in nuclear division. Since these cells are multinucleate (McIndoo, 1939) estimates more than 15 nuclei in one cell), it is possible that the groups of centrioles may have resulted from nuclear division. However, no nuclei were found which showed signs of division. A possible explanation for the occurrence or all these different organelles may be that the muscle cells have not fully differentiated into the classical muscle cell form. The reason for this could be that they would not be able to fulfil the role as a valve if they were long strap-like cells. The differentiation process would have to proceed to a small extent to allow the formation of the few myofibrils. The large areas of the cell not occupied by myofibrils is rather similar to the cytoplasm of myoblasts which form imiginal muscles in Calliphora (Crossley, 1965). But it must be remembered that there are many cases of orifices being controlled by sphincters, which are made up of usual

type muscle cells. The reason why the spherical form of muscle cell is employed in this case is obscure.

The muscle cells are innervated by granule-containing axons. There is a growing body of evidence which shows that the heart of arthropods has this type of innervation, although other types of nerve endings may be present (Komuro, 1970; Johnson, 1966a; Miller and Thomson, 1968; Normann, 1965). Neuromuscular junctions from other groups of animals also have dense granules within the terminals (Atwood et al., 1971). The nerve axons, in their preterminal parts, contain dense granules which are very similar to the small dense neurosecretory granules which have release sites adjacent the haemolymph. This similarity may be fortuitous, but also illustrates the possibility that one particular type of neurosecretory granule can have a dual function, or alternatively, that functionally different granule contained active factors can have similar ultrastructural morphology.

The junctions of the granule-containing axons with the muscle cells have all features of a true synapse. The post-synaptic membrane is also specialized, having a layer of dense material, which is striated periodically, in the synaptic cleft. A similar specialization has been found in other arthropod neuromuscular junctions (Komuro, 1970; Osborne, Finlayson and Rice, 1971; Osborne, 1970; Anderson and Smith, 1971; Smith and Sacktor, 1970). The

function of this post-synaptic membrane specialization is not known, but may be involved with reception of transmitter substances. The origin of the synaptic vesicles was not elucidated in this neuromuscular junction, but may be a consequence of exocytosis (see Smith, A.D., 1971 for review, but also see Osborne, 1970 for a different interpretation).

The occurrence of large numbers of dense granules in the preterminal axon raises the question of the chemical nature of the substance contained therein. The heart wall also has axon terminals similar to those in the valve, so one may speculate from the reactions of the heart to various drugs. Biogenic amines, known to be contained within vesicles, have a marked effect on the heart (Davey, 1964; Miller and Metcalf, 1968). Perhaps it is one of these compounds which acts as a transmitter substance in the neuromuscular synapse of the valve cell as well as the heart. Of course, this does not rule out the possibility of other known or unknown transmitter substances (Osborne, 1970).

The question as to whether the valve has multiterminal and/or polyneuronal innervation is difficult to answer. No longitudinal sections of the axons were found which could indicate that the terminals are anything other than uniterminal, but through chance alone, no truly longitudinal sections may have been cut. Polyneuronal innervation could be present, since up to six terminals have been

found on one muscle cell, but if ultrastructural criteria are sufficient, then it would appear as if all the terminals carry the same transmitter. There does not appear to be any axonal branching within the valve structure itself, and the bundles of axons outside the valve carry about six axons. Whether there is axonal branching in other parts of the neuron(s?) can only be guessed. If all the axon terminals on one muscle are the same type, then it would be reasonable to expect that they all have a common origin since this would be the simplest manner by which contraction of the myofibrils could be initiated. Going further, most of the muscle cells may be innervated by one or a few neurons so that their activity can be synchronized.

The anatomical position of the perikarya which send axons to the valves is not known. The ganglion cells of the LNC do not appear to be synthesising dense granules, so a local position can most probably be ruled out. However, the presence of neurosecretory perikarya have been postulated in the LCN from electrophysiological studies (Miller, 1968; Miller and Usherwood, 1971), but the ultrastructural evidence is not very convincing (Miller and Thomson, 1968). The preterminal parts of the axons contain a few granules, and quite a number of microtubules. This perhaps indicates a rapid transport of granules along the axons to accumulate in the preterminal regions. Rapid

transport of intra-axonal material has been shown in other animals, possibly mediated by microtubules (Smith, D.S., 1971; Dahlström, 1971). If the granules are rapidly ~~bourne~~ borne away from the perikarya, then there may not be any significant build up in the cell body. This would make it difficult to decide whether or not a particular cell body was producing dense granules which accumulate at the terminal only. However, it does seem to be becoming more evident that all types of neurons, including cholinergic ones, produce at least some dense granules which are transported down the axons (see review by A.D.Smith, 1971).

The close spacial disposition of synapses and myofibrils fully supports the controlling function of nerves over muscular contraction. The pathway of signals from the synapses to the myofibrils is most probably directed through the sarcoplasmic reticulum, since this has a close relationship with the post-synaptic membrane in many cases. The transverse tubular system, on the other hand, only appears to be developed where there is a fairly well organized group of myofibrils which is a relatively long distance from the sarcolemma. This lack of well developed TTS would be in keeping with the slow nature of the muscle contraction in visceral muscle in which graded responses are produced by nervous stimulation

rather than all-or-none responses as in fast skeletal muscle (Osborne, 1970; Anderson and Ellis, 1967; Smith, 1968).

Valve muscle cells are attached to the heart wall by intercalated discs. These discs, or interfibrillar junctions, have similarities with some other types of cardiac intermuscular contacts (e.g. Homarus, Anderson and Smith, 1971; Limulus, Sperelakis, 1971; Palinurus, Baccetti and Biliardi, 1969b), but is simpler than others (e.g. vertebrate heart, Dewey, 1969; Hyalophora, Sanger and McCann, 1968; McCann and Sanger, 1969), in that it does not possess septate junctions or gap junctions. These latter structures, or nexuses, supposedly form pathways of low electrical resistance which allows coupling between adjacent muscle cells. However, Anderson and Smith (1971), found that these closely opposed membranous structures were essentially absent from Homarus cardiac muscle, yet there is a certain amount of electrical coupling. They suggest that the intercalated disc of this type is intermediate between a mechanical attachment site and an electrical pathway. It is doubtful if there is any electrical coupling between the heart wall and the valve muscles in Periplaneta SBV, since these contract 180^{o} out of phase with each other and any coupling would upset this function. In this case, the interfibrillar junction probably acts as

mechanical attachment device. This is supported by the fact that a "half" interfibrillar junction was found adjacent strands of extracellular connective tissue. Attachment between individual valve muscles seems to reside in the interdigitated, infolded regions of the cells and no specialized zones are present. These regions are also strengthened by strands of collagen-containing connective tissue. These infolded regions are dove-tailed together and would not be expected to come apart. Also, they form the pathway for the axons and small tracheae, so parting at this junction would most probably upset essential functional links.

The oscillatory function and non-function of the valves to limit haemolymph flow for various periods of time was also noticed by McIndoo (1939). He also noted the dissimilar rates of function of adjacent and opposite valves (actually he thought it was the contraction of the vessel itself, but this is not possible since there is no muscle in the SBV wall). How this is controlled and the reason for this type of function is obscure. Perhaps it is a result of isolating the heart from the rest of the animal. This is difficult to test since the SBV's are very hard to find by looking through the dorsal cuticle of the intact animal. The persistence of a particular rhythm in the opening and closing of the valve may indicate some sort of local control. If an external controlling path-

way was severed, then either an erratic operation or no operation at all would be expected. Thus a method by which this local "programmed" function is achieved remains a mystery at this stage.

Several possible reasons why the valves operate in this manner can be suggested. Considering the width of the animal, circulation into the entire lateral region at one time may be impossible. If particular valves are closed, then this would allow haemolymph to be pumped more efficiently through those vessels whose valves are open. Alternatively, the internal organs are at different levels of the body. Some organs may require a greater amount of nutrient than other organs at particular times. This could be achieved by pumping a greater proportion of the haemolymph to these organs relative to the amounts being pumped elsewhere.

Edwards and Challice (1960), in their study of the dorsal vessel of Blattella germanica recognised dense areas in the dorsal diaphragm. These areas are most probably cross-sections of collagenous strands similar to those found in Periplaneta (this work). Similarly, Smith (1968) showed the structure of the dorsal diaphragm but made no comment on the strands of connective tissue embedded in the basal lamella. Perhaps this was due to the staining methods used, since these strands are best shown when phosphotungstic acid is used as a block stain. Collagen is not usually formed into strands as in the dorsal diaphragm and segmen-

tal blood vessels, but usually occurs as randomly oriented fibres or laminated sheets (e.g. as in the neural lamella or the heart of Locusta (Hoffmann and Levi, 1965)). The diameter of individual collagen fibres in the SBV's is not as great as in other parts of the body (e.g. Harper et al., 1967). Perhaps this related to the special function which the connective tissue has to fulfil in the dorsal diaphragm and SBV's. These structures are highly elastic and the parallel arrangement of fibres probably allow this flexibility. Also, there does appear to be differences in the type of collagen found in different insects (see review by Ashhurst, 1968), and perhaps the small diameter collagen fibres of the SBV's is slightly different from other types.

The similarities in structure of the dorsal diaphragm and the SBV's would suggest that the latter are most probably specialized derivatives of the former. Certainly, the dorsal diaphragm does not have neurosecretory axons running across it and it is a somewhat thicker structure than the SBV walls, but the basic similarity is quite evident. This specialization of the dorsal diaphragm to form blood vessels allows circulation of haemolymph into the lateral regions of these large insects. While several other groups of arthropods (mantids and myriopods) have segmental blood vessels (McIndoo, 1939; Nutting, 1951), the structure of these vessels are of myoepithelial type (unpublished observations) and could perhaps be derivatives of the heart wall

rather than the dorsal diaphragm.

The structure of the SBV fibroblasts is similar to those found in vertebrates (e.g. Porter, 1966). Their cytoplasmic ramifications are perhaps more extensive than that found in other cases, but the form of the endoplasmic reticulum is most distinctive. The tortuous nature of the cytoplasm is perhaps a reflection of the type of connective tissue which it produces. Fibroblasts of a somewhat more compact form constitute the medullary cells in the rectal papillae of Calliphora (Gupta and Berridge, 1966 and the connective tissue layers associated with the heart of Locusta (Hoffmann and Levi, 1965). In this case, the endoplasmic reticulum dilations contain fibrous material which is banded in a periodic manner (see also photographs in Ashhurst, 1968 and Smith, 1968). It would appear that the collagen fibres are formed within the endoplasmic reticulum and the fibres reach the exterior by deterioration of part of the cytoplasm. In the fibroblasts of the SBV's, a few dilations of the endoplasmic reticulum contain fibrous material, but there does not appear to be any periodic banding. On this slight evidence, it is suggested that the collagenous strands are synthesised within the endoplasmic reticulum of the fibroblasts.

The neural lamella of the ventral nerve cord of insects contains collagen fibres and these are produced by sheath cells (see Ashhurst, 1968). Wigglesworth (1959)

suggested that the perineurium be regarded as a constituent of the neuroglial system; a single cell type differentiates into specialized cells of different types. However, Pipa (1961) indicates that there is no information on the embryological derivation of the perineurium and glial tissue. In this respect, it is interesting to note that cells which encircle axons in the distal portions of the SBV's also have characteristics of fibroblasts in other parts of their cytoplasm. In these cases, it is difficult to classify the cells as either glial or fibroblast. Also, glia and fibroblasts has the same general form, except that glia forms sheaths around axons and fibroblasts form ramifying layers. It would appear that glial tissue and fibroblasts as well as the perineurium belong to a more general class of tissue and that their different functions are reflected in slight differences in their ultrastructural features.

Since the collagenous strands are fully formed in adult animals, the amount of synthetic activity within the fibroblasts would be expected to be limited to "maintenance" processes only. Also, if collagen is formed directly in the endoplasmic reticulum, then the function of the numerous Golgi bodies remains to be explained. Perhaps they produce mucopolysaccharides which constitute the basement membrane, since this is believed to be produced locally (in the neural lamella at least, Ashhurst, 1968). However, Beaulaton (1968) maintains that haemocytes are responsible

for the formation of the basement lamella (at least in the prothoracic gland). The Golgi bodies in the SBV fibroblasts produce a few vesicles but there is no apparent release of these vesicles to the exterior. If Golgi bodies are involved in the formation of the basement lamella, then it is perhaps not surprising to find little activity in fully formed tissue. The situation may be quite different in actively growing tissue.

Another enigmatic structure of the fibroblasts is the occurrence of bags of microvesicles on the surface of the cell. They seem to show a sequence of formation and breakdown. The vesicles initially form a small bag on the surface of the fibroblast which then forms into a bag with a constricted neck. The vesicles never appear to be present deep within the fibroblasts. The final stage is a balloon in the plasma membrane with a few vesicles around the inner perimeter. The means by which the vesicles are lost from the bag is probably by rupture in the bounding membrane since some of these balloon-like structures is problematical. There is a reference to similar structures in the rabbit ovary (Espey, 1971). The fibroblasts forming the thecal layer of the ovary produce these bags of microvesicles during post-ovulation. Their function is supposedly to release a substance to break down the extracellular ground substance to allow remodelling of the ovarian thecal layer. If this hypothesis is correct, then the connective tissue

of the SBV's may be undergoing continual reshaping and replacement. The SBV's and the dorsal diaphragm undergo continual low amplitude stretching and relaxation in concert with the heart beat. This persistent mechanical straining may cause malfunction of the elastic components which would require continual maintenance. So perhaps it is a feasible proposition that the fibroblasts are continually removing and replenishing the extracellular connective tissue.

Structures similar to microvesicular bags have been described by Vegge (1972), in capillary endothelium. These structures were more common in tissue fixed with hyperosmotic fixative and were thought to be artifacts. In the cockroach SBV fibroblasts, structures such as mitochondrial cristae and the nuclear membranes, disruption of which are usually good indicators of osmotic damage during fixation, remain well preserved. The fibroblasts do not appear to be actively controlling movement of material as found in capillary endothelium, so it is doubtful if their plasma membranes would be particularly sensitive to osmotic effects.

Neurosecretory Axons in the Segmental Blood Vessels

The SBV's of the cockroach heart fulfil a role as neurohaemal organs as well as arteries. The neurohaemal regions are extensions of the neurosecretory system assoc-

iated with the lateral cardiac nerves (Johnson, 1966a). The methylene blue technique shows the origin of the SBV axons from the lateral cardiac nerves and the acridine orange method confirms the neurosecretory nature of these axons. The naked axons, being in contact with blood moving away from the heart, most probably release neurosecretory material which affect organs in the general body cavity. The similarities of the types of elementary granules in the LCN's and SBV's would indicate that the material contained within them serve similar functions and are not limited to controlling cardiac function and the pericardial cells (Johnson, 1966a). One organ which is most probably affected by NSM's from this sytem is the fat body since this is one of the first organs to come into contact with arterial haemolymph. Also, the distal ends of the SBV's are merely channels through the fat body. The increase in the incidence of release sites during the night period when these insects are most active would indicate that the NSM's are closely involved in daily activity changes. The fat body, being a synthetically active and a storage organ, would necessarily be involved in such daily activities and could well be controlled by the neurosecretory system of the heart and SBV's.

Other bodily functions cannot of course be excluded, since the haemolymph reaches other tissues besides the fat body on leaving the SBV's. Also, other tissues have

diurnal rhythms in their function. For instance, the Malpighian tubules ramify through the posterior portion of the abdomen and even penetrate into the heart by way of the incurrent ostia and SBV's (unpublished observations).

The problem of control of organs can be considered from another angle. The well established neurohaemal organs, such as the corpora cardiaca, have been shown to affect many systems. Also, there is a growing amount of data on the systems which are affected by the perisympathetic neurohaemal organs associated with the ventral nerve chain. These two neurohaemal organs, as well as the LCN's, are known to cause changes in the heart rhythm. It would appear that neurohaemal organs contain material with a broad spectrum of activities. However, the bioassay techniques usually use isolated preparations in vitro and this does not necessarily agree with the actual control of the test preparations in vivo. It would seem to be an uneconomic system that requires control from three different neurohaemal organs such as the evidence is for neurohormonal control of the heart.

The neurosecretory systems of the entire animal may be inherently inefficient, being duplicated in each body segment and with fusion of some segments in the head to give a more important role to the corpora cardiaca (Finlayson & Osborne, 1968). This multiplication of neurohaemal structures reflects the primitive status of blatterian insects, and is not so evident in higher insects (such as Hemiptera and Diptera), where there is a high degree

of fusion of the nervous system.

An alternative argument for the multiple control of heart function may involve the origin of the stimulus which invokes changes in heart rate. For instance, stimuli received by the antennae or mouthparts may only be translated in the corpora cardiaca, while stimuli received by the anal cerci may only be translated by the ventral nerve cord neurohaemal organs or the lateral cardiac nerve system. This type of control also has elements of inefficiency. A single system which translates all incoming stimuli which require a change in heart rate as a necessary response would be the most expedient method for control. Also, the neurohaemal system fulfilling this role would be best placed near the effector organ. In relation to the heart, the LCN neurohaemal organ would appear to be the most suited system for cardiac control. The reasons why other neurohaemal organs affect the heart are not entirely clear, but perhaps the other functions which they control require a concomitant increase in heart rate. Alternatively, all neurohaemal organs may release a neurohormone which increases the general activity of all systems, the heart being only one of these systems.

The classification of neurosecretory granules into three morphological types seems to be justified even though Johnson (1966a) recognises only two types in the LCN. The third type (large electron-lucent) was mentioned by Johnson (1966a), but he classified the types of granules on size and

not on electron density since he considered the pale granules may be due to variable fixation. However, variable fixation is hard to justify since adjacent axons in the SBV show each separate granule type and it is difficult to find a reason why axons which have such a close proximity to each other can be fixed in different ways. Also, there is a slight size difference in these two types of granules, the paler being larger than the dense granules. Miller and Thomson (1968) recognize this pale type of granule as well as a fourth, electron transparent type. This latter type was only found in the lateral cardiac nerve and the segmental nerve from the ventral nerve cord (see chapter on peripheral neurosecretory system in the abdomen). Distinction of different types of neurosecretory material by using a variety of morphological characteristics has beem employed by many workers (e.g. Andrews et al., 1971), and provides a consistent means by which differentiation can be attained.

Most of the perikarya of the neurosecretory axons are in the ventral nerve cord. The axons loop around the body and join up in the LCN's and some then proceed laterally again down the SBV's. From the position of the neurosecretory axons in the SBV's, it would appear that they are not immediately involved in the control of the heart. The question arises as to why these neurosecretory axons should make such a circuitous journey before they release their material. There must be some advantage in such an arrangement,

since there is already a neurohaemal system close to the ganglia. The site of action of the released NSM's and the effectiveness of the location of the release sites must be amongst these advantages.

Mechanism of Release of Neurosecretory Material

The origin of small vesicles in both synaptoid and synaptic areas has been clarified recently (Smith, U., 1970; Bunt, 1969; Douglas et al., 1971; Nagasawa et al., 1971; Holtzman et al., 1971). By the use of tracers (Ferritin, horseradish peroxidase, Thorotrast), it has been shown that many of the small vesicles have at some time been in contact with the axolemma since the tracer is contained within the small vesicles. It would appear as if either the small vesicles are derived from the axolemma, or that the small vesicles momentarily fuse with the membrane long enough to take in the tracer. This fusion is thought to be the means by which the contents of the vesicles are released to the exterior of the axon. While these processes may not account for all the small vesicles present in the axon terminals, it would appear as if it is a common event.

Holtzman et al., (1971) have shown that the number of vesicles containing tracer increases if the particular axon terminals are electrically stimulated. The maximum increase is shown a few minutes after the cessation of electrical stimulus. These results have been interpreted as a recovery process following the increase in membrane surface

area by fusion of the vesicles with the axolemma. This is also in agreement with calculations showing the increase in membrane surface area due to additions made by vesicle fusion (Bittner and Kennedy, 1970). An alternative method of membrane retrieval has been postulated by Douglas et al., (1971) and Nagasawa et al., (1971). Coated vesicles are produced at axon terminals of the posterior pituitary and these are thought to reduce the increase in surface area. The coated vesicles soon lose their coating and becom indistinguishable from synaptic vesicles. Bunt (1969) has found coated vesicles in the sinus gland of the crayfish Procambarus clarkii, and also speculates on the formation of "synaptic" vesicles from these coated vesicles.

Coated vesicles are though to be involved in protein uptake (Roth and Porter, 1964; Bowers, 1964). The coating of polygonal basketwork material surround the vesicle is instrumental in the mechanism of infolding and fission of the membrane (Kaneseki and Kadota, 1969). Perhaps coated vesicles in neurosecretory axon terminals serve two functions, firstly, to retrieve axonal membrane as already mentioned, and secondly, to take up protein from the immediate vicinity. The protein taken up could be the carrier protein of the neurosecretory material since this substance would have completed its function once the active factor has reached the exterior. As Thorn (1970) points out, the ratio of vasopressin to neurophysin is much higher in the external medium than in the tissue

of the neurohypophysis. If the neurophysin is retrieved by the coated vesicles soon after it is released, then this would account for the variable ratios of vasopressin to neurophysin. Coated vesicles can probably take up particular proteins such as in the case of oocytes (Roth and Porter, 1964).

The fate of coated vesicles appears to involve decoating (Douglas et al., 1971) to form small vesicles. These small vesicles may aggregate into tubular formations as postulated by Wendelaar Bonga (1971) for small vesicles derived from NSM release. These tubular formations are apparently transported proximally. Alternatively, the small vesicles may undergo transformation into scyphosomes which have been found in the neurosecretory axons of the SBV's and LCN's of the cockroach.

The presence of scyphosomes or similar structures in neurosecretory axons does not appear to have been previously reported. The only structures which seem to have a similar form have been found by Holtzman and Peterson (1969), but in this case the cup-shaped bodies are much larger (ca. 250nm compared with 100nm in the cockroach), and are more irregular in shape. Also, the contents are quite dense whereas in the cockroach they are usually electron-lucent with only a few having dense contents. Holtzman and Peterson (1969) suggest that these bodies fuse with multivesicular bodies which are later digested by primary lysosomes. No evidence for the fate of scyphosomes was found in the cockroach material.

However, a sequence of formation of these bodies by a progressive fusion of small vesicles and transformation of the membrane shape does appear to be present in some axon terminals. The reason why a single surface (topological sense) should take on a cup shape is obscure. A spherical form would seemingly have the least surface forces involved. If the inner surface of the membrane possessed a high surface tension compared with the outer surface, then a scyphiform structure may be attainable. How such a surface tension could be generated could well be the result of some substance (carrier protein?) contained within the membrane.

There is a growing body evidence that many secretory produces are released from the cell by a calcium-dependent exocytotic process (Smith, A.D., 1971), and it now appears probable that this is the major method by which membrane-bound material is transported across the plasma membrane. The evidence for this process in the release of NSM is well supported in some cases (e.g. Nagasawa et al., 1970; Smith and Smith, 1966; Bunt, 1969; Normann, 1965, 1969; Weitzman, 1969), but there is little morphological evidence in others (e.g. Scharrer, 1968; Shivers, 1969; Johnson, 1966a,b; Krisch et al., 1972). In the cases where exocytosis has not been found, other methods of release have been postulated. These involve the fragmentation of neurosecretory granules into small vesicles prior to their release. However, the small vesicles do not have the same electron density as the

NS granules. Also, the number of cases in which this type of process occurs appears to be relatively small since most published electron micrographs showing proposed release sites fail to reveal this fragmentation. A more difficult situation is the release of material from the large dense granules from axons in Periplaneta (Johnson, 1966a). In this case, the cluster of small vesicles adjacent the plasmalemma usually have a density equal to that of the neurosecretory granules. This would be more in keeping with the fragmentation of the granules. However, the paucity of cases showing budding-off of small dense vesicles from the neurosecretory granules does not lend support to this hypothesis.

These cases may perhaps be explained if the time course of the exocytosis process is variable from species to species. As Normann (1969) suggests, the time taken for the actual release of the contents of a neurosecretory granule may be very short, thus accounting for the small number of omega figures. The clusters of small vesicles are more stable and hence more of these are found. Also, the integrity of the released neurosecretory material appears to be great in Calliphora (Normann, 1965) and in Carausius (Smith and Smith, 1966), since extruded granules remain identifiable after fixation and other preparative procedures. However, in the peripheral neurosecretory axons of Carausius (Finlayson and Osborne, 1968) and in Periplaneta (chapter

in this thesis), the neurosecretory granules rarely show themselves after release and only an indentation or omega figure remains as evidence for exocytosis.

Normann (1970) found that, by statistical analysis, the vesicle clusters or "synaptoids" would be present even when exocytotic omega figures are absent. A further point he made was that the number of omega figures in relation to vesicle clusters increased markedly by depolarization of the terminals with either acetylcholine and eserine or high-potassium saline. This was interpreted as a modification of the stimulus which releases NSM. Electrical stimulation of the brain is more akin to the natural stimulus than total and continual depolarization of the terminals. This type of stimulus increases the number of omega figures in the axon terminals. These findings give some clue to the local events which allow release of NSM. Depolarization of the axon membrane appears to be an essential step in the release phenomenon.

A comparison of the form of release sites in insect neurohaemal tissue is interesting. Those cases where exocytosis is quite evident, the small vesicles do not appear to form into synaptoid configurations while those in which do not have convincing evidence for exocytosis show a synaptoid structure. The question arises as to whether or not these different forms represent the same process. Electrical stimulation gives rise to release of NSM's (e.g. Kater, 1968;

Normann and Duve, 1969; Cooke, 1967), and morphological changes in the neurohaemal organs coincides with this release (e.g. Scharrer and Kater, 1969; Normann, 1969). However, the morphological types of release still remain as apparently two different types, but they are both induced by a similar mechanism. As pointed out by Normann (1970), the actual technique used may determine whether or not exocytotic omega figures are found. This part of the process may have a very short time interval.

A similar enigma is reached in the mode of release of acetylcholine from synapses. While there is evidence for exocytosis at the physiological level, the morphological evidence does not appear to support this contention. While the evidence for a general mechanism (exocytosis) for the release of active factors is well documented (A.D.Smith, 1971), the seemingly different modes of release of neurosecretory material in different insects remains difficult to comprehend. More work is obviously needed to solve this problem.

The nature of release sites or "synaptoid" junctions (Scharrer, 1968), has many similarities with the presynaptic side of true synapses. The layer of dense material associated with the neurosecretory release sites, suspected of being free NSM by Scharrer (1968) and Johnson (1966a, b), is very similar to presynaptic dense material of true synapses.

An alternative explanation of the presence of this dense material in release sites may be that it is an integral component of the exocytotic process. Undoubtedly, there must be some specialization of the membrane area to allow exocytosis, independent of the nature of the material being released. These specialized presynaptic and synaptoid junctions are then merely manifestations of a similar process. Whether or not there is a postsynaptic specialization depends on the function of the effector cell, being necessary in some (e.g. axon/skeletal muscle) but not in others (e.g. axon/anterior pituitary).

The general problem of release of active factors (either neurotransmitter or neurohormone) from nervous tissue most probably has underlying basic mechanisms. The morphological form of the sites of release could well be related to the function of the material being released and the form and function of the target tissue. In the case of axo-axonal and cholinergic neuromuscular communication, the function of the target tissue requires a message which induces an almost immediate response. A true synapse allows these conditions to be fulfilled. A specialized area of the presynaptic axon is immediately opposite a specialized site of the postsynaptic target tissue. This site most probably carries receptor sites which are senitive to the neurotransmitter. In this manner, a precise and exact signal can be relayed from one cell to another. A similar situation exists

in neurohaemal organs where axons containing small dense granules (B fibres) synapse onto axons containing large dense granules (A fibres), (Scharrer, 1969, 1970 for reviews).

In situations which do not require such precise control, such as vertebrate smooth muscle, a less organized arrangement of axon and target tissue is found. The naked axon terminals are separated from the effector by a gap of ca. 400nm and there is no specialization of the postsynaptic membrane. Presumably, the entire surface of the target tissue is sensitive to the transmitted substance. Similar situations are found in insect tissues where presumed neurosecretory axons are associated with different organs (e.g. muscle, Finlayson, Osborne and Rice (1971); spermatheca, Gupta and Smith (1969); rectal papillae, Gupta and Berridge (1966); epidermis, Maddrell (1965)). The corpora allata are innervated by neurosecretory axons (e.g. Scharrer, (1964), Johnson (1966b)), and the gap between the axon and corpora allata cells is quite narrow. Sometimes, there does appear to be some specialization of the effector cell membrane (Johnson, 1966b), and perhaps represents a situation more similar to a true synapse than just a close contact type of relationship.

The third broad category of axonal/effector cell relations is shown by the neurohaemal organs. Here, the effector organs are at a distance from the sites of release

of active factors from the nervous tissue. The type of signal transmitted is of a long-term variety. In consequence, the effector tissue is stimulated over long periods of time to allow it to fulfil its long term function. The manner in which the effector responds is also important. Such processes require sustained control since they are not "instantaneous" events (e.g. protein synthesis).

The division of active factors into two distinct and sharply different groups, neurotransmitters and neurohormones, appears to be too restrictive. A looser classification, considering the function of effector organs, would add a more unifying concept to nervous control. The type of control required is reflected in the chemical nature of the released substance as well as in the location and morphology of the release sites. Short-term active factors (such as acetylcholine) are small molecules capable of inducing a rapid reaction in the effector cell. The initial and main step in the reaction is a membrane phenomenon (i.e. depolarization). The active factors are rapidly deactivated by enzymes located at the release sites (true synapses) and can be recycled back into the nerve terminal to become available for reactivation in readiness for retransmission. Long-term active factors (posterior pituitary hormones of vertebrates, some NSM's of invertebrates, on the other hand, are large molecules (polypeptides). These are likely to be more resistant to enzymatic deactivation <u>in vivo</u> and can

induce long-term reactions in target tissues which are situated at a distance from the release site. These reactions require the main effect to be within the cell and not just associated with the plasmalemma, although this would be the initial site of reception of the active factor.

It would appear that the type of transmitter employed for a particular effector function is closely linked with the type of function which the effector fulfils. The morphological arrangement of the stimulator and the effector is also a reflection of the type of function of the effector. Thus, at one end of the scale is the classical synaptic junction while at the other end of the scale is the release site of a neurosecretory axon which is adjacent a vascular space. While all neurons appear to have a secretory function (A.D.Smith, 1971), this is perhaps not a good criterion for unifying all types of neurons under "neurohumoral" as was done by De Robertis (1964). The value of neurosecretion as a concept is still valid (Scharrer, 1970), although the demarcation of neurosecretory neurons is not as sharp as previously thought. The occurrence of types of neurons which secrete material which is neither truly neurohumoral not truly neurohormonal (Scharrer, 1969, 1970), perhaps only strengthens the idea that some neurons are more specialized and are able to synthesize, store and release pep-

tidergic materials. The physiological function of this released material, while desirable to know, is not necessary to the concept of neurosecretion. The definition of neurosecretion incorporating physiological function (Knowles and Bern, 1966), reflects the next and possibly the most useful stage of investigation of neurosecretory phenomena. While this has been achieved in a few cases, there are many instances where there is only cytological evidence for the occurrence of neurosecretory material. This aspect should be ~~bourne~~ born in mind in any investigation of neurosecretory phenomena, but is not essential to the concept of neurosecretion.

NEUROSECRETORY CELLS AND AXONS IN THE PERIPHERAL NERVOUS SYSTEM OF THE ABDOMEN

INTRODUCTION

The knowledge of neurosecretory systems in insects has increased greatly over the last few years (Maddrell, 1969). Axons containing neurosecretory granules in the peripheral nervous system have been described on numerous occasions, but there are only two reports so far of the occurrence of neurosecretory (NS) cell bodies outside the central nervous system (Bowers and Johnson, 1966; Finlayson and Osborne, 1968). In Carausius (Finlayson and Osborne, op. cit.), the NS neurons are mainly associated with the median/transverse nerves (which also contain the perisympathetic neurohaemal organs) and a "link" nerve which connects peripheral nerves of adjacent segments. Also, there is a neurohaemal area associated with the nerves around the spiracular region. In the aphid Myzus persicae, there are glandular cells along the course of the lateral nerve from the corpora cardiaca (Bowers and Johnson, 1966). These cells appear to send processes along the lateral nerve.

This chapter describes a neurosecretory system in the abdomen of Periplaneta americana. The thoracic region was not investigated.

MATERIALS AND METHODS

Adults and late instar larvae of P. americana were used. For whole mounts of the particular region of the nervous system, animals were vitally stained with reduced methylene blue (Pantin, 1946) and fixed in situ. Later, the required region was dissected out and mounted. The nervous tissue was also vitally stained with acridine orange to locate neurosecretory material (Beattie, 1971a; also see chapter in this thesis). Other material was sectioned and stained with either paraldehyde fuchsin (Ewen, 1962) or a modified azan technique (Hubschman, 1962). For electron microscopy, the tissue was fixed in situ with ice-cold 3% glutaraldehyde (pH 7.3, 0.1M phosphate buffer, plus 7% sucrose), post-fixed in 1% osmium tetroxide and embedded in Epon 812. During the dehydration step, the tissue was blocked stained with saturated lead acetate in 70% alcohol. Although this staining technique gave reasonably good contrast, it also gave an electron-dense deposit. This deposit occurred mainly on the inner glial membranes of the nervous tissue. This interesting result seems to mark a boundary around the perimeter of the nerves. Most axons contained within this boundary do not posses NS granules (except some in the link nerve), while the only axons outside the boundary are granule containing axons. This type of deposit is reminiscent of lead salts produced in various histochemical enzyme reactions. However, the nature of the reaction in this

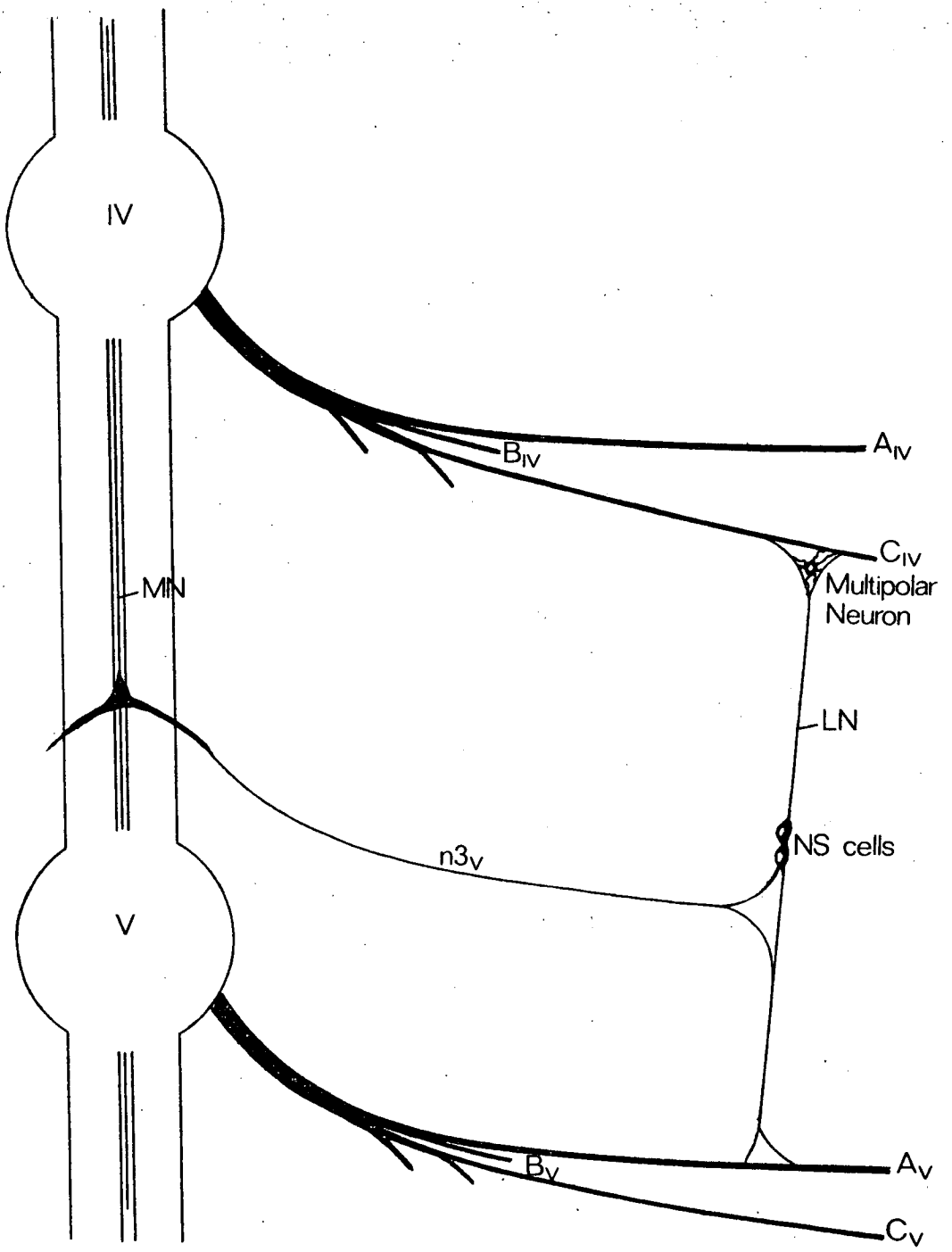
case is impossible to ascertain.

The gross anatomy of the peripheral nervous system appears to be best described by Shankland (1965). The observations made during this work agree very closely with Shankland's description which is in contrast to other reports summarized by Guthrie and Tindall (1968). For this reason, Shankland's (1965) terminology will be used with his C3 being called the link nerve because of its anatomical importance (Finlayson and Osborne, 1968).

RESULTS

Anatomy of the Lateral Nervous System

Although Shankland (1965) describes considerable variation in the arrangement of nerves associated with the median-transverse nerve (n3), the most consistent form found during this work is shown in Text Fig. 1. At each junction, the nerves usually form a triangular arrangement (Figs. 1a, 1b, 2a), where the various nerve tracts join or depart from each other. There is a pair of neurons on the link nerve usually near the junction of n3 and the link nerve (Figs. 1b, 2b, 2c). These neurons appear to be bipolar but the processes are very fine and difficult to distinguish. At the junction of the link nerve and nerve C of the preceding segment, there is a multipolar neuron (Fig. 2a). There are at least 8 processes arising from this neuron. The processes run out along all of the nerve tracts meeting at this junction. Some of the processes are fine and stain uniformly



TEXT FIG. 1. Schematic diagram of part of the ventral nerve cord and peripheral nerves showing the location of peripheral neurons. LN - link nerve; MN - median nerve with neurohaemal organ; $n3_V$ - transverse branch of median nerve; NS cells - neurosecretory cell; Capital letters - major nerves of each segment; Roman numerals - abdominal segments. Terminology after Shankland (1965).

while other processes are coarser and have granular contents. Sometimes these latter processes are varicose and branching (Fig. 3a).

Neurosecretory Tracts

Using the acridine orange technique, the NS tract are readily identified. A small number of NS axons are present in nerve A proximal to the junction with the link nerve. At this junction, most of the NS axons pass into the link nerve and a few bypass the link nerve and continue distally. A larger tracts of NS axons joins nerve A from the link nerve and apparently pass distally towards the dorsal side of the body (Fig. 4a). However, from these anatomical studies, it is not possible to determine the direction in which the NSM is travelling. It is assumed that the direction of movement is away from the central nervous system.

The link nerve contains large amounts of NSM. The NS axons are derived from the median/transverse nerve, nerve C and intrinsic neurons as well as from nerve A. The paired intrinsic neurons contain NSM (Fig. 2c), but the contents of the multipolar neuron is unknown, mainly because this was discovered much later than the paired neurons and its reaction to acridine orange was not investigated.

Nerve C receives NS axons from the link nerve. These axons appear to run distally towards the spiracular region.

The segmental nerve which joins the lateral cardiac nerve contains much NSM (Figs. 3b, 4b). This segmental nerve is derived from nerve A. The NSM is abundant near the heart, but less evident in the nerve in its more lateral portions where it passes ventral to the dorsal longitudinal muscles.

Staining Characteristics of the Paired Neurosecretory Neurons

The paired NS neurons stain green with the paraldehyde fuchsin technique and the cytoplasm contains a few small purple granules. These granules may be NSM but could also be lysosomes (see ultrastructure section). Using azan, the cytoplasm is yellow and contains a few red granules. Likewise, these granules may be NSM or lysosomes.

The multipolar neuron was not investigated.

Ultrastructure of the Paired Neurosecretory Neurons

These neurons are situated superficially on the link nerve and are surrounded by a very thin layer of glial tissue (Figs. 5, 6a). Outside the glial layer is a sheath of connective tissue 0.5-1.5 µm thick. This layer contains a few collagen fibres. The plasmalemma of the NS cells is generally smooth but occasionally glial invaginations do occur.

The cytoplasm of these cells contain abundant rough endoplasmic reticulum which is poorly organized (Fig. 6a).

There are numerous Golgi bodies which appear to be active (Fig. 6b). The Golgi cisternae are dilated. This is possibly due to osmotic shock during primary fixation. Similarly, the mitochondria are poorly preserved and this also suggests osmotic damage. Neurosecretory granules are sparsely distributed throughout the cytoplasm (Figs. 5, 6a, 6b) and have a mean diameter of ca. 210nm. Care should be taken with this measurement, considering the poor preservation of the mitochondria and Golgi bodies. There are often large lysosome-like bodies in the neurons (Figs. 5, 6b). They are usually associated with the Golgi bodies.

The multipolar neurons were not studied at the ultrastructural level.

Ultrastructure of the Nerve Tracts

Nerve A. This nerve contains small NS axons around its perimeter (Fig. 7). Occasionally, the NS axons have local dilations (Fig. 8). The "glial barrier" produced by the staining technique is well shown in Figure 8 and illustrates the external position of the NS axons. Quite often the NS axons have no glial sheath, and the basement lamella (neural lamella) is directly adjacent to them.

Nerve C. This nerve has a few NS axons which have a similar distribution to the NS axons in nerve A.

The Link Nerve. This nerve is composed of a main bundle of axons and scattered axons which lie in the thick neural

lamella (Fig. 9). The main bundle has at least one large axon which has a well developed glial sheath (Fig. 9), and does not contain any granules. This is in contrast to most of the other axons in the main bundle. These are smaller, have moderate amounts of glial tissue surrounding them, and possess a few to many dense granules (Fig. 9). The dense "glial barrier" surrounds the main bundle of axons. The axons in the peripheral portion of the nerve do not have well developed glia. Some are partially or totally devoid of glial tissue so that they have naked areas immediately adjacent the basement lamella (Figs. 9, 10a, 10b).

There appear to be three types of NS axon in the link nerve. Some contain large dense granules (ca.150nm diameter) (Fig. 10a), others contain small dense granules (ca. 90nm diameter) Fig. 10a), and yet others which contain almost transparent granules (ca. 150 nm diameter) (Fig. 10b). Some of these axons in the peripheral part of the nerve have small vesicles as well as granules in the axoplasm (Figs. 10a, 10b). These small vesicles probably indicate release sites for NSM. The three types of granule-containing axons are also found in the segmental nerve close to the junction with the lateral cardiac nerve (Fig. 11). The transparent granule type is quite rare, there being only one or perhaps two profiles in any part of the link nerve. The occurrence of this axon type near the heart perhaps indi-

cates a pathway for this particular axon type. The other types of NS axons may also follow this pathway.

DISCUSSION

The majority of NS neurons occur in the central nervous system of insects and have processes which carry the secretory material to a neurohaemal organ which is closely associated with the central nervous system. Only recently has there been reports of neurons possessing neurosecretory features in the peripheral nervous system (Finlayson and Osborne, 1968; Bowers and Johnson, 1966). The transverse branch of the unpaired median nerve of Carausius and Phormia have NS neurons associated with it and these are located distally to the perisympathetic neurohaemal organs (Finlayson and Osborne, 1968).

In Carausius, there are about 10 neurons associated with the peripheral nerves of one side of each abdominal segment. The 4 neurons on the link nerve as well as some of the others show cytological features of neurosecretory material. They stain positively with paraldehyde fuchsin and show elementary granules at the ultrastructural level. Their size is variable and depends on the degree of distention with NS material. In Periplaneta, the peripheral neurosecretory cells on the link nerve are constant in their dimensions and only contain a few paraldehyde fuchsin positive granules. This staining may not be neurosecretory material since there are numerous lysosomes in these neurons

which are also stained by paraldehyde fuchsin. Similar variations in staining of NS material is shown in the perisympathetic neurohaemal organ of different insects (Brady and Maddrell, 1967). While staining characteristics are variable in different species, the ultrastructure of NS material is more consistent in form. In both Carausius and Periplaneta, the peripheral neurons contain electron dense elementary granules which are produced by Golgi bodies. Similarly, in both species the NS neurons are superficially placed on the nerve tract and have a thin glial envelope separating the neuron cell body from the haemolymph.

In both Carausius and Periplaneta, the fine structure of the link nerve is similar. There are axons deep within the nerve which have many glial layers around them and the axoplasm is devoid of dense granules. Other axons in the core of the nerve have lesser amounts of glial tissue but contain granules. The peripheral axons of the link nerve of both species are at least partially free of glial tissue and show evidence for the release of NS material. Since the structure of the link nerve is similar in both species, then the nerve probably serves a similar function in both. The existent of neurohaemal tissue in this region of the body may indicate that the target tissue of the NS material is also in this vicinity.

The greatest proportion of NS axons in the lateral region of the peripheral nerves is contributed by the median/

transverse nerve, although a smaller number is contributed by the major nerves. The actual axonal pathways are almost impossible to trace since individual axons cannot be identified. However, judging from the amount of NS material demonstrated by the acridine orange technique, it would appear that the median/transverse nerve carries the most NS axons and these pass into the major nerve tracts. It appears doubtful if many of the few axons leaving the ventral ganglia by nerve A go past the link nerve. On the other hand, NS axons are contributed to nerve A from the link nerve. Similarly, the majority of NS axons in the distal part of nerve C are derived from the link nerve. The contribution of axons from the peripheral NS neurons is difficult to estimate since the processes from these neurons could not be distinguished. These cells do not appear to be producing large amounts of NS material, but it may be transported rapidly from the perikarya and there is not build-up of NS granules in the cell body. The pathway of some axons reaching the lateral cardiac nerve does appear to be fairly clear. The transparent granules type of NS axon is quite rare. It is found in the link nerve and in the segmental nerve near the junction with the lateral cardiac nerve. Brady and Maddrell (1967) have shown that a similar type of NS axon is present in the perisympathetic organs. Also, this type of axon was not found in the major nerves proximal to the junction with the link nerve. It would seem possible that at least some of the NS

axons contained in the median/transverse nerve continue outwards and contribute to the lateral cardiac nerve. The perikarya of these axons may be some of those nerve cell bodies supplying the median nerve which is located in the midline of the abdominal ganglia (Smalley, 1970).

Since the transparent granule type of axons appear to travel along the transverse branch of the median nerve to join the main dorsal nerve (nerve A) via the link nerve, this pathway may also be the route for other NS axons found in the lateral cardiac nerve. Also, the link nerve and the lateral cardiac nerve contain similar morphological types of granule-containing axons, but this does not necessarily provide positive evidence since axons containing similar granules are found in many parts of the nervous system.

The fact that the perisympathetic organs have a cardio-accelerator factor in them (Raabe et. al., 1966), supports the view that some of the NS axons which reach the heart may come from the median system. Although this factor may be released from the perisympathetic organ itself, it could well be transported through this organ to the heart. This would give a more localized and effective distribution of the cardioaccelerator.

Many neurons associated with peripheral nerve tracts are sensory receptor cell bodies (see review by Finlayson, 1968). Since motor neurons have their cell bodies in the

central nervous system and the sensory neurons have their's in the periphery, Finlayson and Osborne (1968) argue that the peripheral NS cells are derived from sensory neurons and suggest that the neuron may be functionally reversed. An alternative novel explanation may be that the secretory activity of the peripheral NS cells may be regulated by external stimuli which the NS cell itself receives. This would be independent of the central nervous system and would allow a local control of NS neurons. This could allow control of adjacent tissue by the NS cells in response to local stimuli.

The lateral NS system associated with the link nerve is approximately half-way between the perisympathetic organ and the lateral cardiac nerve, both of which are neurohaemal organs. It would appear that this neurosecretory tract has neurohaemal organs along its length. Whether or not these different neurohaemal organs release the same type of NS material is not known, but there does seem to be a certain amount of restriction of at least some types of NS axons (e.g. the transparent granule type is restricted mainly to the perisympathetic organ). The present indications are that NS cells and axons are widespread and must be of great importance to the physiology of the insects.

DEGENERATING ABDOMINAL MUSCLES

INTRODUCTION

The insect body undergoes marked and sometimes extensive modification during metamorphosis. External changes are paralleled by changes in almost all internal systems (Whitten, 1968). Endopterygote insects undergo more obvious and more drastic changes than exopterygote insects in which structural changes are progressive throughout the larval stages but more prominent after the final moult. The skeletal musculature shows many and varied modifications which usually reflect the changes in the modes of locomotion as well as changes associated with the moulting process itself.

Flight muscles, which are present only in adults, often degenerate after a dispersive phase. This degeneration is often associated with reproduction (Johnson, 1957; Edwards, 1969; Bhakthan et al., 1970) or diapause (Stegwee et al., 1963). Some degenerate flight muscles are capable of regenerating (Bhakthan et al., 1971; Stegwee et al., 1963). Intersegmental muscles of the abdomen, which are involved in the moulting process, often degenerate either in the pupa or young adult (Finlayson, 1956; Lockshin and Williams, 1965a; Crossley, 1968). In Rhodnius, intersegmental muscles undergo a cycle of formation and involution at each moult (Wigglesworth, 1956).

The control of flight muscle degeneration is dependent on hormones present in the haemolymph (Johnson, 1959; Stegwee et al., 1963; Edwards, 1970). The cyclical degeneration and regeneration of intersegmental muscles in Rhodnius is not controlled by nerves and was assumed to be affected by a hormone (Wigglesworth, 1956). Similarly, Crossley (1965, 1968) maintains that the nervous system is not involved in intersegmental muscle degeneration of Calliphora, and that phagocyte invasion was dependent on ecdysone titre and premature degeneration could be induced by crustecdysone. In various Lepidoptera, Finlayson (1956) found that intersegmental muscles could be induced to degenerate precociously by denervation, but removal of classical endocrine sources had no effect. However, Randall (1970) found that degeneration of the proleg retractor muscle of Galleria was induced prematurely by denervation and this process was accentuated by injection of 20-hydroxyecdysone.

In a series of papers Lockshin and Williams (1964, 1965a-d), investigated the control mechanisms for degeneration of intersegmental imaginicaducous muscles of silkmoths. The muscles are potentiated for degeneration during early adult development. This process involves the synthesis of cytolytic enzymes induced by ecdysone. These enzymes are stored in lysosomes until the pupa moults. On emergence of the adult, the lysosomes are triggered by a cessation of neural input to the muscles and the muscles degenerate within

two days. The process of activation of the lysosomes depends on synthesis of RNA and proteins (Lockshin, 1969). The nature of the neural trigger is of a conventional transmitter type and neurotrophic or neurosecretory processes are not involved (Lockshin, 1971). This trigger is supposedly the cessation of input, but depends on synaptic transmission. This conundrum is yet to be resolved, but it may depend on a special type of input a short time prior to cessation of nervous activity.

Runion and Pipa (1970) challenge the view that the cessation of nervous activity induces cytolysis. They found that neural activity continued during muscle degeneration. The trigger mechanism could still be neural if it happened to be a special type of stimulus. Runion and Pipa (1970) measured all neural input, but did not distinguish between the type of axons carrying impulses.

This chapter describes the degeneration of three pairs of skeletal muscles in the lateral regions of the pregenital segments of the abdomen of Periplaneta. These muscles have not been described previously.

MATERIALS AND METHODS

Adults of known age and larvae were used. The muscles were fixed in situ with 3% glutaraldehyde in phosphate buffer. They were then either dissected and lightly stained with haematoxylin, or studied with phase contrast optics, without staining, or processed further for electron microscopy.

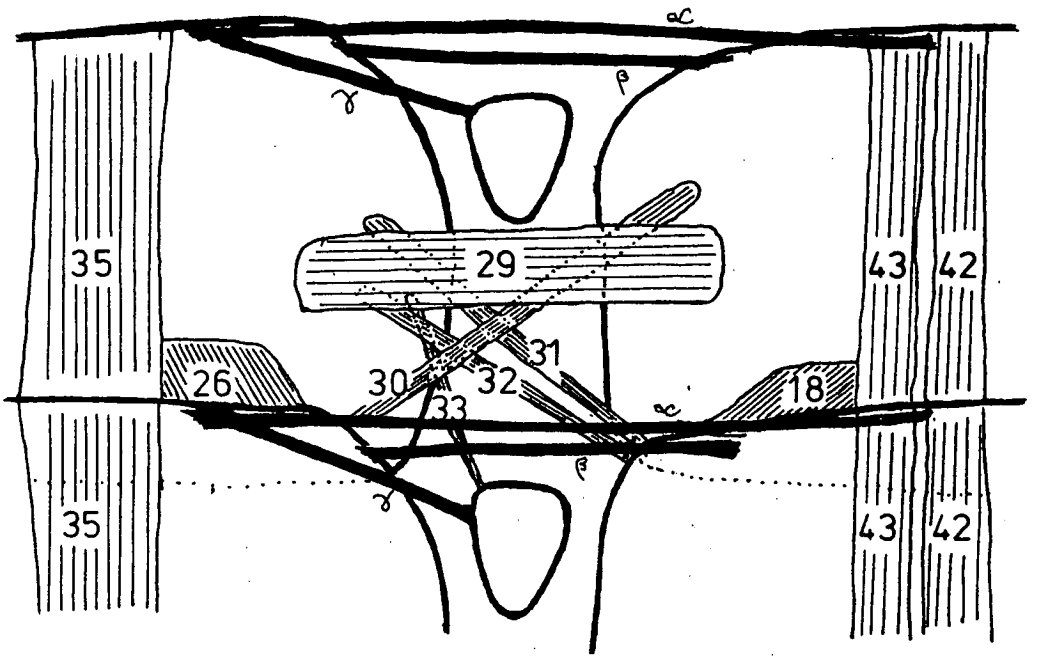
For muscle terminology, the numbered system proposed by Shankland (1965) is used (Text Fig. 1). Greek letters are used to identify the degenerating muscles, since Shakland's (op. cit) scheme, and other schemes, does not easily lend itself to being extended to include these degenerating muscles without changing the numbering order of other muscles.

RESULTS

Anatomy of the Degenerating Muscles

The three pairs of degenerating muscles are located in the lateral region of the segments. In the nymph, all these muscles are approximately the same size(ca. 250/um diameter: - i.e. smaller than muscle 32, but larger than muscle 33). The muscles are strap-like and fan out slightly at their attachment to the cuticle. These muscles attach to the sclerites of the same segment. The first has its origin on the anterior border of the sternite just lateral to the median internal ventral (intersegmental) muscle (muscle 34) and the insertion is on the anterior border of the tergite near the 4th median internal dorsal (intersegmental) muscle (muscle 42). From its attachments, this muscle will be called the secondary sterno-tergal muscle, since this distinguishes it from the large sterno-tergal muscle (muscle 29) (α in Text Fig. 1).

The second muscle has its insertion on the anterior lateral corner of the sternite just lateral to the insertion


α
β
γ
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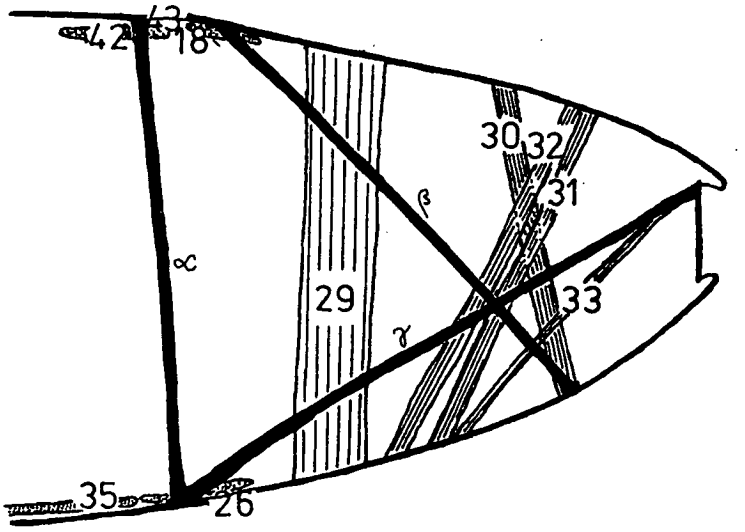
A.


42
18
30
32
31
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26

B.

of muscle 30 (intersegmental tergo-sternal). The origin is on the anterior border of the tergite of the same segment just median to the insertion of muscle 31 (anterior intersegmental sterno-tergal). This muscle will be called the tertiary sterno-tergal muscle (ϱ in Text Fig. 1).

The third muscle has its origin immediately lateral to the origin of the secondary sterno-tergal muscle. The insertion is on the anterio-ventral aspect of the pleurite of the same segment, ventral to the insertion of muscle 33 (intersegmental sterno-paratergal or intersegmental sterno-pleural). This muscle will be called the sterno-pleural (or paratergal) muscle, (γ in Text Fig. 1).

The secondary sterno-tergal muscle may be innervated by a fine branch from the link nerve (or C3). The source of innervation of this muscle was never definitely found, but occasionally the small nerve mentioned above could be found in whole mounts of the link nerve and its associated nerve fibres.

The sterno-pleural muscle receives a small nerve from nerve C which arises a short distance distal to the link nerve (C3).

The tertiary sterno-tergal muscle was discovered much later than the other two degenerating muscles. However, muscles 30-33 receive branches of nerve C and considering the close anatomical position of these muscles to the tertiary sterno-tergal muscle, it too may be innervated by a branch from nerve C.

Degeneration of Muscles

In adults, the three pairs of muscles appear to degenerate at the same rate. The secondary sterno-tergal muscle was studied in detail, and the other two muscles probably follow a very similar pattern.

The rate of degeneration is slow, taking about three weeks for all muscle fibres to disappear. The whole muscle does not degenerate synchronously, and the individual fibres undergo lysis relatively independent of each other. Also, there appears to be a certain amount of regional difference within each fibre. The muscles from different segments do not degenerate at the same rate, the more posterior ones being slowest.

The muscles are about 250/um in diameter in the larvae, but in the 14 day adult they are ca. 45/um (Fig. 1). After three weeks, the muscles have no fibres remaining. The "empty" connective tissue sheath is maintained as a strand between the attachments of the degenerate muscle (Fig. 2b). When the whole muscle is viewed with phase contrast, the integrity of the fibres can be fairly well judged (see Figs. 1a, 1b, 2a, 2b). In some cases, the fibres are equally degenerate (Fig. 1a), but in others a single fibre still maintains a healthy condition (Fig. 1b). The degenerative process appears at the Z-line level initially (Fig. 2a), and reveals itself as a cluster of granules. This is probably a result of formation of dense bodies associated with the

mitochondria (see later). Fibres which have lost all orientation of myofilaments appear as tubular structures containing a few dark granules as well as refractile granules (Figs. 1b, 2b). Nerve tracts appear as dark, varicose lines (Figs. 2a, 2b). The tracheae do not degenerate, and are very obvious in degenerated muscle. They serve as a useful marker in dissection for locating degenerate muscles.

At the ultrastructural level, the process of degeneration can be followed fairly closely even though individual fibres show different degrees of degeneration. In one day old adults, the fibres are not particularly different from larval muscle (Fig. 3). The only difference may be a reduction in the amount of sarcoplasmic reticulum. The fibres are ca. 10/um in diameter. In 6 day adults, the fibres are much smaller (ca. 5/um diameter - i.e. about $\frac{1}{4}$ the cross-sectional area), and the amount of stromal material between the fibres is relatively increased (Fig. 4). Also, the fibres are more circular in profile and the transverse tubule invaginations are wider (compare Fig. 3 with Fig. 4). The organization of filaments shows marked changes. In 1 day animals, the primary filaments appear to be well preserved and the irregular orbits of secondary filaments (up to at least 12) do not appear to be disturbed. There are occasional empty spaces between the filaments as well as

around the perimeter of the fibres (Fig. 5a), and these may represent initial degenerative processes or perhaps they are fixation artifacts. In 2 day and 3 day adults, some of the mitochondria show vacuolization and are often associated with dense bodies (Fig. 5b). These dense bodies appear to arise from the mitochondria. Other mitochondria, which are not associated with dense bodies, swell and the matrix between the cristae becomes less dense (compare Fig. 5a with Fig. 6). Occasional membranous structures are present in fibres of 6 day adults (Fig. 6). The most obvious feature of 6 day adult fibres is the disorganization of the myofilaments (Fig. 6). Most of the thick filaments have disappeared, leaving the thin filaments as misoriented arrays. By day 10, the individual fibres are small (2-3 μm) and rarely show any sarcolemmal invaginations (Fig. 7). Also, there are no diads as in day 6 adults. From day 10 onwards, the fibres are often difficult to recognise as muscle cells. Many have large autophagic vacuoles or cytolysosomes filled with dense membranous material (Fig. 7). These cells are usually surrounded by processes from phagocytic cells. Other fibres contain huge multilaminar bodies, similarly surrounded by phagocytic tissue (Fig. 8). The phagocytes contain dialated cisternae of rough endoplasmic reticulum and active Golgi bodies as well as large membranous structures (Fig. 9). Coated vesicles are fairly common in the phagocytes. Occasion-

ally, phagocytes are found in younger adults. In these cases, they appear to surround a muscle fibre which has undergone lysis (Fig. 10). Other fibres surrounding the degenerate muscle fibre have normal constituents for an adult of that age.

The axons in the nerves supplying this muscle appear to be of two or perhaps three different types. The axon bundles running between the muscle fibres usually have well developed glial sheaths (Fig. 11). Quite a few of the axons have large dense granules (ca. 160-200nm diameter), while others are devoid of granules. The number of granules shown in a particular axon would depend on the level of the section, since the granule population is low. Other axons, peripherally located in the bundle, are naked and contain many large dense neurosecretory granules. These axons show evidence of release of neurosecretory material (Figs. 12a, 12b). Other axons in the bundles contain small dense granules (ca. 90nm diameter) and small vesicles (30-40nm diameter) (Fig. 12a). These latter axons may be pre-terminal parts of neuromuscular junctions, since the synaptic regions contain a similar population of granules and vesicles (Figs. 11, 13a). On very rare occasions, synapsing axon terminals are without dense granules.

The contents of the presynaptic compartment of neuromuscular junctions do not appear to change during degeneration of the muscle fibres. Synaptic junctions are fully formed in 1 day adults (Fig. 13a), but as the animal

ages the close association of axon and muscle widens. In old animals (more than three weeks), the muscle fibres have completely degenerated, but the nervous system and tracheae remain (Figs. 13a, 13c). In these cases, all the axons contain some sort of granular or vesicular material. The granule-containing axons are often naked to the stroma. The granules are small (ca. 90nm). These naked axons can be interpreted in two ways. They could either be neurosecretory axons at a release area, or presynaptic axons which have lost the post-synaptic components. Glial tissue surrounding the axons does not contain many organelles and the plasma membranes are irregular. This may be a result of disuse of the nerve axons and the tissue may be functionally inactive.

DISCUSSION

Caducous muscles are found in many insects and their disappearance is associated with a new phase in the life cycle of the animal. The speed at which muscles degenerate depends upon the species, tending to be shorter in insects which have a short adult life span and longer in those which have a relatively long life span. For example, the intersegmental muscles of the lepidopteran Antheraea pernyi degenerate within two days (Lockshin and Williams, 1965a), while the intersegmental muscles of Rhodnius involute in 10 days (Wigglesworth, 1956). Similarly, the flight muscles of Dysdercus take about 5 days to disappear (Edwards, 1969),

while flight muscles in Aphis fabae take more than 14 days (Johnson, 1959). The degeneration of abdominal muscles in Periplaneta take about 3 weeks and this correlates with the long life span of adults of this species.

Irrespective of the rates of degeneration, the phases of muscle breakdown are fairly similar in the species which have been studied ultrastructurally (e.g. Ips, Bhakthan et al., 1970; Antheraea, Lockshin and Williams, 1965a; Gallaria, Randall and Pipa, 1969). The case of Periplaneta americana agrees with the previously reported cases. The degenerative process depends on autolytic action of lysosomes within the muscle cells. In the case of Calliphora (Crossley, 1968), there is little evidence of disruption of the myofilaments by autolytic lysosomes, and degeneration proceeds by haemocytes phagocytosing fragments of relative intact muscle. However, Whitten (1964) found that muscles in Sarcophaga and Drosophila undergo fragmentation and loss of striation prior to invasion by phagocytes.

Phagocytes are not evident in all degenerating muscles. In the cases where a particular set of muscles can undergo cyclical degeneration and regeneration, phagocytes are not found during degeneration. Other cases of irreversible degeneration, phagocytosis is found in some (e.g. Crossley, 1968; Randall and Pipa, 1969), but not in others e.g. Edwards, 1969; Bhakthan et al., 1970). An intermediate cases has been reported (Lockshin and Williams, 1965a), where

phagocytes invade the degenerating muscle only during the terminal processes of disintegration. The muscles in the lateral abdominal region of Periplaneta show obvious signs of phagocytosis. Phagocytosis would seem to be an efficient method by which degenerating or foreign tissue could be effectively removed and eliminated. Perhaps in the cases cited above where phagocytosis is not evident the degree of invasion of the muscle tissue by phagocytes is limited. The phagocytes may only attack muscle fragments once they have left the muscle as such, and do not infiltrate the muscle. This may reflect the amount of autolytic digestion within the muscle. Some muscles, such as in Antheraea (lockshin and Williams, 1965), have well formed lysosomes present in functional muscle. The lysosomes are triggered and digest the muscle. Phagocyte only appear much later. The reverse appears to be the case in Calliphora (Crossley, 1968). Phagocytes actively invade apparently healthy muscle which show no signs of autolytic degeneration. From these examples, it would appear as if there is a balance between autolysis and phagocytosis.

The neuromuscular junctions in degenerating muscles of Periplaneta do not appear to degenerate, at least in a morphological sense. This is contrary to the findings of Randall and Pipa (1969) on Galleria proleg retractor muscles where synaptic organelles clump together and become dense and where myelin figures appear. These latter features are

somewhat similar to the morphological appearance of axons which show degeneration by axon severence. Even in the completely degenerate cockroach muscle, the nervous tissue maintains its integrity, although not in a similar condition as in functional muscle.

Part of the nervous tissue present in completely degenerate muscle may consitute stretch receptor dendrites. Their structure has some similarities to other cockroach stretch receptors (Osborne, 1963). It may be argued that since these muscles appear to play a role in the moulting process, then it might be expected that there would be stretch receptors associated with them. The muscles are placed in a position where they could affect reshaping and alignment of the soft cuticular sclerites once the old cuticle has been cast off and the abdomen is still distended. Output from stretch receptors in these muscles would contain information about the extent of realignment of the sclerites. In adults, these postulated stretch receptors could still act in a capacity of registering the amount of deformation of the abdominal sclerites with respect to one another.

Certainly, all the nervous tissue remaining in the degenerate muscle is not of this type. Much of it appears to be non-functional motor terminals since the axons contain synaptic vesicles and small dense granules. Other axons have many characteristics of neurosecretory release sites.

The neurosecretory axons found in the nerves

to this muscle would appear to have some functional association with the muscle. The axons penetrate between the muscle fibres and are not in direct contact with circulating haemolymph. Toselli and Pepe (1968) illustrated axons which appear to be neurosecretory in the ventral abdominal muscles of Rhodnius, but made no comment on them. Maddrell (1967) also noted these axons as well as neurosecretory axons in the tergo-sternal muscles. He proposed that the neurosecretory axons of the intersegmental muscles may be involved in the degeneration and regeneration of these muscles. However, Wigglesworth (1956) showed that transection of nerves to these muscles did not alter the regeneration/degeneration cycle. Neurosecretory axons have been found in muscles of other insects (Osborne, Finlayson and Rice, 1971), and a trophic role has been postulated for them. This trophic role of NSM may be necessary for the maintainence of muscle (Finlayson, 1960), but has been denied by Lockshin (1971). The function of neurosecretory axons in degenerating muscles as well as muscles in general, remains tentative and requires a much closer examination.

VASCULAR SYSTEM SUPPLYING THE ANTENNAE

INTRODUCTION

Cephalic pusatile organs would appear to be necessary for circulation of haemolymph in the antennae. They have been found in orthopteroids, Ephemoptera, Odonata, Hymenoptera, Lepidoptera and Diptera (see review by Jones, 1964). This is a broad spectrum of insect orders and perhaps cephalic accessory hearts are of general occurrence in insects. There is a lack of data in this regard. However, the problem of pumping haemolymph up a narrow extremity, such as an antenna, would seem to require a pump and vessel closely associated with the extremity. Increased hydraulic pressure produced by the heart would not achieve circulation since this would not allow return of haemolymph. Similar problems are found in the wings, legs and posterior (cercal) appendages. Wings and legs have been shown to have accessory hearts (Jones, 1964).

Antennal circulation in orthopteroids is possibly the best described amongst the insects. Pawlowa's (1895) description includes a comprehensive study of Periplaneta orientalis L.. A brief summary of this work will be given as an outline of the gross structure and function of this part of the vascular system.

The system consists of two ampullae, one on each side of the frons, a muscle connecting the ampullae and

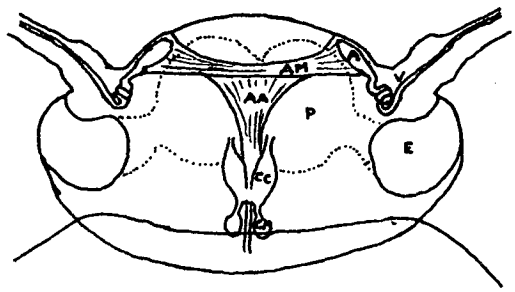
to the antenna (Text fig. 1). The ampullae are situated immediately beneath the cuticle. Their position is marked on the anterior surface of the head by slightly domed, light brown circles which are ventral to the ocelli and median to the antennal sockets. Each ampulla has a valve allowing the entry of haemolymph only and a vessel leading off from the dorsal aspect which serves as the only exit for haemolymph. The antennal vessel is convoluted in the head capsule and has thick walls. The portion of the vessel in the antenna has thin walls and runs a straight course to the tip of the antenna. In its course through the antenna, the vessel has small opening which allow the egress of haemolymph. The horizontal muscle between the ampullae has the anterior aorta attached to it. Also, the horizontal muscle pulsates and activates the ampullae.

Studies of the cephalic pulsatile organs in other insects are less detailed. There has been no fine structural studies made on any of these systems as far as can be ascertained. The present work describes the ultrastructure of the antennal circulatory system in Periplaneta americana. This system has shown itself to be quite complex and is probably involved in more functions than the circulation of haemolymph.

MATERIALS AND METHODS

Adults of both sexes, as well as late larval

stages, of Periplaneta americana were used for electron microscopical studies. Very small larvae were dissected and it was found that the system occurs in early developmental forms. For electron microscopy, the animals were decapitated and the head placed in ice-cold 3% glutaraldehyde fixative. The antennal circulatory system in the head was dissected out and placed in fresh fixative. To study the vessel in the antenna, the antenna from newly moulted animals were cut off and divided into short sections for fixation. All tissue was post-fixed in 1% or 2% OsO_4 and dehydrated in graded ethanol and embedded in Epon 812 according to the usual schedule. Thin sections were stained with uranyl acetate and lead acetate.



TEXT FIG. 1.

Schematic frontal section through the head of P. americana showing the antennal circulatory system (from Pawlowa, 1895). A - ampulla, AA - anterior aorta, AM - ampulla muscle, P - protocerebrum, CA - corpus allatum, CC - corpora cardiaca, E - eye, V - vessel.

RESULTS

The anterior aorta

Anterior to the retrocerebral complex, the aorta loses its tubular shape and forms an inverted "V" beneath the supraoesophageal ganglion. The aorta is open in its ventral aspect and the muscular wall forms a canopy. At its anterior end, the aorta fans out and attaches to the horizontal ampulla muscle. The wall of the anterior aorta is about 7/um thick. The sarcolemma is relatively smooth and does not have regular infoldings at the Z lines as in the posterior portions of the heart (Figs. 1, 2, 3). Correspondingly, there are no lateral projections filled with mitochondria. Instead, the mitochondria are situated in columnar zones between the myofibrils. These zones also contain glycogen granules and sometimes elements of the sarcoplasmic reticulum (SR) and transverse tubular system (TTS). The myofibrils tend to be oriented along the aorta rather than in a circular manner as in the rest of the heart. This orientation is not strictly adhered to, and the myofibrils in one cell may be almost at right angles to one another. The Z lines are distinct, being placed at 4-6/um intervals. There is a dense plaque of material on the sarcolemma opposite the Z line (Fig. 3). These appear to be hemidesmosomes. The sarcomere length is somewhat longer than that found in the heart (2-3/um). Narrow I bands can be

indistinctly differentiated from the long A bands. There does not appear to be an H band present in this muscle. The SR only partially divides the filaments into myofibrils. It does, however, occur in, or on both sides of, the Z line. The SR forms diads with the TTS in the region of the A band. The filaments are organized on the usual slow muscle or visceral plan. Each thick filament is surrounded by about 12 thin filaments.

Accompanying the anterior aorta are numerous axons which are superficial to the muscle. These axons probably arise from the corpora cardiaca since most of them contain neurosecretory granules. The axons fan out anteriorly and at least some of the axons reach the ampulla muscle. The axons can be divided into three types. Firstly, there are axons of ca. 2/um diameter which are well sheathed with glial tissue (Fig. 4). These axons contain many microtubules and scattered profiles of smooth endoplasmic reticulum. Very few granules are found in these axons (but see figure 3 where part of the axon contains many granules while the other part is almost devoid of them). Secondly, there are axons which contain a mixed population of granules (Fig. 4, 5). The granules vary in size from 100nm to 220nm and vary in electron density from almost clear to quite dense. The clearer granules cover the whole size range, while the

dense granules tend to be of a medium size. These axons tend to occupy a peripheral part of the nerve bundles and only have a thin glial layer in places. Some of these axons abut the basement lamella directly and occasionally exocytotic omega figures are found (Fig. 4). Thirdly, there are axons which contain a fairly homogeneous population of dense granules (Figs. 2, 5). These are about 140nm in diameter. These axons also tend to occupy peripheral regions. The glial sheath of these axons, like that of the other type of neurosecretory axon, is sparse. Quite often, the axons are naked to the haemolymph. In these naked areas, there are often accumulations of small vesicles which have medium-dense contents. They vary in size from 30 to 50 nm. Their shape is variable, some being spherical while others are elongate to almost tubular.

The Horizontal Ampulla Muscle

This muscle produces the force which synchronously operates the ampullae. Its fine structure raises questions concerning the distribution of different types of muscle fibres within one muscle, the nature of its innervation, and its lateral attachment to the ampulla wall. At least some of the axons associated with the muscle form a neurohaemal organ at the inner surface of the ampulla wall.

The muscle fibres are roughly polygonal in cross-

section (Fig. 6). Each fibre is surrounded by a thin basement lamella. The more regular shaped fibres are ca. 10/um across, but some fibres are flattened and measure ca. 15/um by 5/um. Nerves and tracheae are present in amongst the fibres, usually in the space between 3 or more fibres. Nuclei are situated laterally in the cell. Long spindle-shaped nuclei, up to 25/um in length, are usually found in fibres which have long sarcomeres (see later). Shorter and broader nuclei are found in fibres with short sarcomeres. There is some overlap in these two forms of nuclei associated with the different sarcomere length fibres.

In transverse section of the fibres, the thick filaments show a hexagonal arrangement. They have a density of about 300/um^2. Each thick filament is surrounded by up to 12 thin filaments (inset of Fig. 6). The thin filaments form an orbit around the thick filaments. No distinction in the filament organization of the different types of fibre could be found. This is made difficult because the differences are only obvious in longitudinal section.

A, I, and Z bands are present in the myofibrils, but other bands are not evident. The I bands appear to be narrow, but this would depend on the degree of contraction of the fibres at the time of fixation. Not withstanding

this variability, there are apparently two types of fibres which can be differentiated on the length of the sarcomere. One type has a sarcomere length of ca. 3/um (Fig. 7a), while the other type has a length of ca. 6/um (Fig. 3, 8). The length of the A band is ca 2/um and 5/um, respectively. The long sarcomere fibres tend to form a peripheral group around the more central short sarcomere fibres.

The TTS appears to be different in the two types of fibres. In the long sarcomere type, there are cleft-like sarcolemmal invaginations at the level of the Z lines (Z tubules) (Fig. 8). Flattened tubules, at least some of which arise from the Z tubules, run longitudinally between the myofibrils and form diads with the SR in the A bands. Also, sarcolemmal invaginations at other levels of the sarcomere (mainly at the A bands) give rise to flattened tubules which form diads with the SR. Whether these two components of the TTS are continuous, remain unproven. In the short sarcomere fibres, Z tubules are rare. Instead, invaginations with square to rectangular cross-section penetrate the fibres at the A bands (Fig. 7b). Diads are formed either on these invaginations, or on flattened tubules of the usual TTS.

The SR is fairly extensive and divides the fibres into myofibrils. However, the SR is variable in its

development, and often the cisternae form an attenuated single line only (Figs. 3, 6, 7a, 7b, 8). The myofibrils are sometimes triangular, but mostly rectangular in transverse section. No filaments would be more than 0.25/um from the SR.

The diads formed with the TTS occur at two levels in the A band of the long sarcomere fibres (Figs. 3 and 8). These are at about one quarter of the length of the A band from each end of the A band. The short sarcomere fibres have diads at a similar level, but they do not have the same regularity of the long sarcomere fibres.

The SR and TTS are separated by a gap of ca. 25nm at the diads (Figs. 7a, 7b, 8). The gap contains dense material. This material is often accumulated on the SR side of the gap and it has periodic projections extending towards the TTS membrane. These projections have a fairly consistent spacing. Some projections appear to be two-membered, the pair lying very close together.

The RS is usually accompanied by mitochondria and glycogen granules (Figs. 3, 6, 7a, 7b, 8). Glycogen granules also often occur around the peripheral margin of the fibres and amongst the I band filaments. The mitochondria are elongate (up to ca. 4/um long) and usually occur in columns between the myofibrils. Sometimes they form pairs on either side of the Z lines. The cristae are usually transverse and dense intramitochordrial

granules are often present between the cristae. The density of mitochrondria can be high in some regions of the sarcomere. At the I bands, mitochondria occupy about 50% of the volume, but only 20% at the A bands.

The Z lines are usually fairly well aligned, but sometimes they are stepped and other times they run irregularly across the fibre. At the zones where the Z lines are adjacent the sarcolemma, whether it is the edge of the fibre or a Z tubule, a dense layer is formed on the cytoplasmic side of the membrane. The dense layer has a matching fuzzy dense accumulation directly opposite on the extracellular surface (Figs. 3, 6, 8). These densities could well be hemidesmosomes. A similar structure is found at the end of the fibres where they abut the ampulla wall (Figs. 9, 10a). At these sites, the extracellular fuzzy layer merges with the connective tissues which forms part of the ampulla wall. Occasionally, the muscle fibres penetrate into the wall and come into close contact with another type of cell (Fig. 10a). These cells appear to be a form of glial tissue since, in some instances, they surround nerve axons. In the regions where they abut muscle cells, the contain microtubules and fibrous elements. The microtubules and fibres are approximately parallel to each other as well as to the filaments of the muscle cell. At

the zone of contact between these cells and the muscle fibre, a desmosome is present. These contacts, together with the hemidesmosomes which are of general occurrence at the fibre terminal, appear to be the only mode of attachment of the muscle fibres to the ampulla wall. The terminal hemidesmosomes are similar to those found in the excurrent ostia valves in the heart of this animal and are also similar to one half of an "interfibrillar" junction (see chapter on segmental blood vessels of this thesis). Apparently, this type of membrane/fibril modification is one type of adhesive device for insect muscle cells.

The microtubule-containing "glial" cells, onto which the muscles sometimes attach, also possess hemidesmosomes on other parts of their membrane (Fig. 10b). In these regions, the microtubules are also accompanied by fibrous material. Again, it appears as if the hemidesmosomes act as an anchoring device.

Nerves Associated with the Ampulla Muscle and the Ampullae

The origin of nerve axons found in this muscle have not been fully elucidated. Certainly, a great proportion are derived from the axons in the anterior aorta. The region in which the anterior aorta and the ampulla muscle are in contact possesses many granule-containing axons. The other source of innervation may be from the nervus

connectivus which runs from the frontal ganglion to the protocerebrum, passing through the ampulla en route. The region in which this nerve penetrates the muscle has not been successfully sectioned, so innervation from this source remains unsubstantiated.

Many axons associated with the ampulla muscle contain electron-dense granules. Those which apparently do not contain granules may be sections through granule-containing axons which has a paucity of granules in the sectioned area. This is exemplified by figure 3. Of course, this does not preclude the existence of granule-free axons. Segregation of the granule-containing axons into sub-classes is difficult because of the small population of granules in any one axon. This is different from the situation in the anterior aorta where there are many granules in each axon.

The axons in the muscle are usually in bundles of up to ten members (Fig. 11). Some axons have two or three layers of glial tissue surrounding them, while others are either partially or totally naked to the haemolymph except for the basement lamella. The glial-invested axons have very few dense granules but the naked axons almost invariably have numerous neurosecretory granules.

Single axons run either between the fibres or on the outside of the muscle (Figs. 12a,b,c). These axons

sometimes have a glial layer partially surrounding them, but others are completely naked. In the majority of cases, where the axons have a naked area, there are small ("synaptic") vesicles present in the axoplasm. These small vesicles are either circular or elongate to tubular in profile. They are about 30nm across. The axons contain a few dense granules which may be neurosecretory granules. Some of these naked axons come into close contact with the muscle fibres. The gap between them is 25-30nm and there is no intervening basement lamella (Fig. 12a). The fibres sometimes have cytoplasmic arms which project from the main body of the muscle cell and are in apposition to the axons. The small vesicles do not appear to form well developed focal areas with associated dense material against the axolemma. Similarly, there does not appear to be any modification of the sarcolemmal surface in the gap between the two types of tissue.

In other cases, the naked axons with small vesicles are separated from the muscle by a gap of ca. 100nm and the basement lamella is present between the axon and the muscle cell (Fig. 12b). These profiles may be slightly more preterminal than the axons first discussed. When the naked axons occur on the outside of the muscle, small vesicles tend to focus on the axolemma. These axons also contain neurosecretory granules. The accumulations of small vesicles perhaps represent release sites of neurosecretory

material (Fig. 12c).

Another type of contact between axon and muscle fibre is present in this muscle. Axons containing a few granules, fairly large amounts of smooth endoplasmic reticulum, mitochondria and microtubules abut the muscle fibres. The gap between them is ca 30nm, lacks a basement lamella, but does not have any other special features (Fig. 13). The adjacent axoplasm does not contain any small vesicles, but sometimes the smooth endoplasmic reticulum forms a sub-membrane cisternae.

At least some of the axons running parallel with the ampulla muscle extend into the ampulla wall beneath the muscle insertion. In this area, large swollen axons, or Herring bodies, are found (Figs. 14, 15, 16). Some of these are greater than 10 µm in diameter. These Herring bodies appear to be pre-terminal since there is an almost-continuous glial envelope surrounding them. This is in contrast to other smaller endings which have the greater part of their plasma membrane in contact with the basement lamella (Figs. 15, 17a, 17b). Also, these smaller endings have different contents to the Herring bodies.

The Herring bodies can be divided into two categories, although there are intermediate forms. The first category contain many neurosecretory granules (Fig. 14, 15). Their density shows the same variation as the granules found

in axons of the anterior aorta (100-250nm and pale to dense). Quite often, there are many mitochondria which are closely packed together. The mitochondria are usually elongate, have fairly well separated cristae, and possess dense intromitochondrial granules. The mitochondrial groups are generally situated in the centre of the Herring body. Occassional microtubules run between the neurosecretory granules. There are few dense bodies and multilaminar bodies in this category of Herring body.

The second category of Herring body has a reduced population of neurosecretory granules which are usually clumped together (Fig. 16). The size and density of the neurosecretory granules are the same as in the first category. There is marked increase in the number of multilaminar bodies, but there does not appear to be any increase in the number of dense bodies. The multilaminar bodies are up to 1.5/um across. The contents of these bodies are variable. Some appear to be empty while others contain groups of vesicles which are about the same size as neurosecretory granules. Yet other multilaminar bodies contain apparently intact mitochondria. The multilaminar bodies are probably autophagic, digesting the contents of the Herring bodies. There are more "free spaces" in this type of Herring body which perhaps suggests autolytic digestion. Some of these spaces are occupied by bundles of microtubules

The overall effect of these spaces is to produce a less dense appearing Herring body.

The axon terminals which are naked to the basement lamella are 1 to 3 µm across and present a different complement of organelles (Figs. 15, 17a, 17b). There are no dense bodies or multilaminar bodies and the number of mitochondria and neurosecretory granules are reduced. The greater part of the axoplasm is filled with small vesicles. The vesicles are spherical or flattened. The flattened form constitutes the major proportion of the vesicle population. They measure ca 60-70nm by ca. 20nm. Some of the vesicles contain dense material. There are no obvious signs of exocytosis of neurosecretory granules although the plasma membrane is often irregular and indented in places. Release sites, with dense focal points, are not evident.

The Ampulla Wall

The wall of the ampulla is composed of a single layer of cells and three extracellular layers on each side of this (Fig. 18). The cells are very irregular and cytoplasmic arms spread out to give a 10-20 µm width between the inner and outer extremities. The cells form a continuous layer except where the muscle is inserted. The adjacent plasma membranes have junctional specialization (Figs. 19a, 19b). Desmosomes (maculae adhaerens types), septate desmosomes and "tight" junctions are present. Thin cytoplasmic arms (down to 0.1 µm thick) sometimes form the total cellular

thickness of the wall (Fig. 18). However, even in these cases, the adjacent plasma membranes are specialized. No case was found where there was an extracellular gap or undifferentiated cell junction.

Irregular, sub-spherical nuclei (ca. 5/um diameter) occur in the wider regions of the cells (Fig. 18). Spherical to ovoid mitochondria (ca. 250nm) are scattered throughout the cytoplasm as are short sections of rough endoplasmic reticulum (Fig. 19a). Some cytoplasmic arms, especially those facing towards the lumen of the ampulla, contain bundles of microtubules (Figs. 18, 19a).

Immediately surrounding the cells is a dense layer of extracellular material (Figs. 18, 21). This layer is finely fibrous and, in places, denser aggregates of these fine fibres are more or less aligned with one another to give sub-parallel arrays. Small accumulations of dense granular material occur throughout this layer. This innermost extracellular layer is perhaps a basement lamella. Its outer edges form an obvious boundary of greater electron density with the next layer.

The middle extracellular layer often has a hyaline appearance, being of an overall less electron density than the epon embedding medium (Fig. 18). It is 1-3.5/um thick. On closer examination, the layer is composed of fine fibres set in a "clear" matrix (Fig. 21). The fibres are not oriented in any particular direction, although small bundles

of fibres are more or less parallel to each other. They do not run a straight course and this gives a swirled effect to the layer. Individual fibres do not have any obvious banding, so they are unlikely to be collagen. Small circular profiles (10-30/um diameter) composed of small dense granules can be seen within this layer. Whether these are discrete units or cross-sections of fibres is not known. This layer is not continuous arround the ampulla. It is absent on the side towards the cuticle. It was impossible to determine the cellular arrangement on this side because the tissue was partially disrupted during dissection. The wall perhaps merges with the hypodermis in this region.

The outer boundary of this layer is delimited by a zone of electron-dense granular material (Fig. 21). The outermost layer is 0.3-0.7/um thick (Figs. 18, 21). It has a density similar to the innermost layer. There does not appear to be any fibres in this irregular layer.

The part of the ampulla wall directly beneath the muscle insertion is different from the other parts of the wall (Figs. 9, 14). In this region, the cellular layer is not continuous as in other parts and is composed mainly of the "glial" tissue which forms desmosomes with the muscle as well as enveloping the axons. This cell type is also similar to the wall cells in that they both contain numerous microtubules. Also, the two cell types appear to merge with one another at the edge of the muscle insertion zone.

There are several other cell types in the muscle insertion zone of the ampulla wall. They are not particularly common, but present some unexpected features. The first of these contain many fine fibres and vesicles (Figs. 14, 20a, 21). In some cases, the plasma membrane appears to be disintegrating. The second type of cell contains concentric layers of membranes which fill the entire cytoplasmic reticulum (Fig. 20b). In places, the membranes form multi-laminar bodies. A third cell type which is difficult to classify is that containing no identifiable organelles with the exception of rare, large vesicles, (Fig. 17b). The remainder of the cytoplasm contains very finely granular dense material. No continuities between these odd cell profiles and any other cells could be found in the material examined. This is partly due to the ramifying nature of the cells in this zone.

Antennal Vessel:- Convoluted Portion In the Head

The vessel maintains a constant diameter in the convoluted portion, but narrows as it enters the antenna. The walls of the vessel are made up of two concentric layers of cells. The inner layer of cells are more or less columnar and the outer layer is flattened. The total thickness of the wall is about 20-25/um, with the inner layer occupying ca. 16/um and the outer layer about 7/um (Figs. 22, 23). The lumen of the vessel is ca. 30/um in diameter.

The inner columnar cells are highly polarized in

form. Their apical (lumenal) surface is highly infolded and irregular. These cytoplasmic projections are usually packed full of parallel microtubules (Fig. 24). Between the microtubules, accumulations of small, dense bodies occur. The microtubules run to the apical ends of the cytoplasm and appear to merge with the membrane. In this region, it is difficult to delimit the ends of the microtubules and the plasma membrane. The junction is obliterated and appears as an ill-defined dense layer. The microtubules run back into the body of the cell and ramify throughout the cytoplasm where they lose their parallel orientation (Fig. 26).

The nuclei of these cells are usually situated in the basal portion (Figs. 22, 23). They are ovoid, measuring ca. 7/um by 5/um. There are prominent nucleoli and these are situated slightly off-centre in the nuclei. Chromatin occurs in clumps around the perimeter of the nuclei. The nuclear membrane is often smooth but, in some cases, it is crenulate on one side. There are numerous, prominent nuclear pores in the membrane.

A large portion of the cytoplasm, especially the region between the nucleus and the base of the apical processes, is packed with mitochondria (Figs. 22, 23, 25). The mitochondria are either circular or elongate in profile. All are most probably ovoid or elongate in form, being ca 1/um long and ca. o.4/um thick in the most extreme form. The

cristae usually run transversely. The material between the cristae is quite dense and contains numerous small, very dense granules.

Rough endoplasmic reticulum occurs in short sections throughout the cytoplasm (Figs. 25, 27a, 27b). Often, it is greatly dialated to give circular profiles. Sometimes these profiles lack attached ribosomes, but are continuous with rough ER with parallel membranes. The contents of the dialated areas are often whispy and fibrous, and sometimes it is an irregular membranous structure. Some of these dialations in the rough ER could be due to osmotic shock during fixation. Free ribosomes, usually in rosettes or clusters occur throughout the cytoplasm of the cell (Fig. 26).

Golgi bodies are present in the basal portion of the cytoplasm (Fig. 26). They are composed of up to five cisternae which have variable density. Few vesicles are associated with the Golgi bodies. In a few cases, fine filaments appear to run between a Golgi body and a centriole (Fig. 27a). Centrioles are often found in these cells, usually in the basal region.

Annulate lamellae are sometimes found in the basal region (Fig. 27b). They are not uncommon. The lamellae are short (just less than 1 /um), and are often continous with rough ER. Stacks of between 5 and 10 lamellae are the most usual form.

Occasional large irregular dense bodies are found in the more basal portions of the cells (Figs. 22, 23). Some are up to 2.5 /um across. In the apical portion and in the apical projections, smaller spherical dense bodies are found. These are ca. 0.5 /um in diameter. The latter dense bodies are distinct from the very small dense bodies which are accumulated amongst the microtubules in the apical projections (Fig. 24). These are only of the order of 70 nm and are often in the form of elongate droplets. Also, their electron density is variable.

The lateral cell membranes are relatively straight. Where adjacent cells are apposed, there are numerous junctional specializations (Figs. 22, 23, 18). "Ordinary" desmosomes (macula adhaerens type) and septate desmosomes are common along the apposed membranes. Also, at least one and usually several "tight" junctions (zonula occludens type) occur between the membranes of adjacent cells. It was difficult to determine whether the latter junctions were truly tight or whether they were gap junctions. From the high magnification micrographs studied, there was evidence for both tight and gap junctions. In all juxtaposed cells examined, these three types of specialized junctions are always present.

The basal membrane is undulating and does not appear to have a distinct basement lamella like the apical

surface (Figs. 22, 23, 26). The apical basement lamella forms a smooth amorphous layer ca. 0.5 µm thick over the ends of the cytoplasmic projections (Fig. 24). The extensive extracellular spaces between the inner and outer layers of cells is filled with bundles of collagen fibres. The matrix between these fibres is sparse.

The basal surface of the inner layer of cells is scolloped in profile. Each cell, where it is free from its neighbours, is domed and thus produces a cleft between adjacent cells. These clefts sometimes run a third of the way down the sides of the cells (Figs. 22, 23).

The outer layer of cells is composed of flattened irregular cells. The cytoplasm is drawn out into many irregular projections. These projections extend to the inner cell basal membranes where they closely adhere at numerous sites (Fig. 22, 23, 26). They also run to the bottom of the clefts between the adjacent inner cells. The junctions of the two different cell types are characterized by desmosomes and "tight" junctions (Fig. 26). The cytoplasmic projections on the outer side of these cells are not so extensive.

The outer cells form an almost-continuous layer, with adjacent plasma membranes closely apposed and usually having junctional modifications. Rarely, is there any extracellular space forming a channel through this layer, so the extracellular spaces between the two layers of cells is

essentially isolated from the haemolymph. The extracellular material surrounding the outer cells is filled with bundles of collagen fibres (Fig. 30). These bundles are mostly oriented in a circular direction around the whole vessel. In some animals, this connective tissue layer has accumulations of dense granular material of up to 0.4/um across (Fig. 30). These dense areas are scattered fairly frequently throughout the connective tissue. The collagen fibres are often packed closer together where they pass around one of these dense areas.

The nuclei of the outer cells are spherical to ellipsoid and are about the same size as the nuclei of the inner cells (Figs. 22, 23). Similarly, there are eccentric nucleoli. There are a fair number of elongate mitochondria throughout the cytoplasm, but they do not show any preferred location. They possess a few dense intramitochondrial granules. A small amount of rough ER is present. There are a few small Golgi bodies which do not appear to be very active. Microtubules are present, but not in great numbers. They tend to be restricted to the cytoplasmic projections. Some of the cytoplasmic projections are filled with dense bodies of variable size and shape (Fig. 29). These small (50-200nm) dense bodies are somewhat similar to those found in the apical projections of the inner cells.

Bundles of tracheae are present on the outermost

surface of the vessel. They do not appear to penetrate into the wall of the vessel. No evidence for the innervation of the cells of the vessel could be found. The nearest nervous tissue which could have some effect on the cells is the neurosecretory terminals in the ampulla wall.

Antennal Vessel:- Portion in the Antennae

In the basal portion of the antenna, the vessel is placed off-centre. Thin cytoplasmic projections of connective (?) tissue radiate out from near the hypodermal layer and meet with the wall of the vessel (Fig. 31). More distally in the antenna, the vessel lies against the thin basement lamella which forms the boundary between the antennal lumen and the hypodermal cells (Fig. 32). There are no cytoplasmic projections of connective tissue supporting the vessel in this region, although an occasional hypodermal cell forms a pillar which abuts the vessel. The vessel becomes progressively thinner as it proceeds outwards along the antenna. In the basal region, the walls are 3-4 /um thick, more distally, it is only ca. 1 /um thick.

The form on the antennal vessel is of a similar nature within the antenna. The wall is only one cell thick and it is not highly polarized as in the convoluted portion in the head. The inner membrane has short cytoplasmic projections, some of which contain a few microtubules. These cytoplasmic projections are only poorly developed in the more distal regions of the antenna. Microtubules are also

found throughout the general cytoplasm of these cells, but never have an organized arrangement. The basement lamella is also much reduced in the antennal region. On the inner surface, it is up to 0.25/um thick, but on the outer surface, it is a very thin diffuse layer ca. 50nm thick. There are no collagen fibres in the outer basement lamella.

The junction between adjacent wall cells have septate desmosomes. This form of cell-to-cell contact is the only specialized type of cell junction apparent. The number of mitochondria is greatly reduced and there is little rough ER. Free ribosomes are fairly abundant in the more proximal portions of the antennal region, but distally they are not so evident. Occasional Golgi bodies are present throughout the wall cells of the vessel in the antenna.

The Investing Layers of Sensory Neurons and Nerves in the Antennae

Groups of sensory nerve cell bodies are situated just beneath the basement lamella boundary layer of the hypodermal cells (Fig. 33). Each group is completely surrounded by a sheathing cell. The sheathing cell has very dense cytoplasm, and this can be traced around the perimeter of the sensory neuron group as a very thin layer (down to 50nm thick). The sheathing cells have small elongate nuclei, well developed rough ER which has wide cisternae, many free ribosomes, few mitochondria and small Golgi bodies.

The very thin cytoplasmic layer does not appear to contain any cell organelles, except where it widens slightly. In these regions, rough ER is often found.

The axons which leave the nerve cell bodies are sheathed by cells which are more characteristic of glial cells. The nuclei of these cells are either between the cell bodies or along the course of the small nerve bundle (Fig. 33). The glial cells do not envelope the nerve cell bodies, but do enclose the axons as they leave the perikarya. The bundles of axons from each group of sensory cells has 2 or 3 glial layers separating them from the haemolymph.

In the main antennal nerves, of which there are two in each antenna, there appear to be two types of glial investment (Fig. 33). Bundles of axons within the nerve are partitioned by a more densely staining type of glia. The bundles possibly arise from single groups of sensory neurons. Around the outside of the nerve a lightly staining type of glia is present. This forms a peripheral layer which has short projections running between the bundles of axons. Axons within a bundle are not isolated from each other by glial tissue and often they come into close contact with one another.

DISCUSSION

The structure of the antennal circulatory system is complex. The implications derived from its structure are important both physiologically and behaviourally, and indicate that antennal sensory reception requires a special internal environment. Cytologically, the system presents some interesting features. The ampulla muscle contains two types of muscle fibre and it is innervated by not-so-well known granule-containing axons. Other axons associated with this system constitute a new neurohaemal system which has some unique features and appears to be intimately involved with antennal function. The antennal vessel is complex, and its structure is similar to other epithelia involved in active transport. The components of the antennal circulatory system will be discussed in turn, although each component forms an integral part of the whole system.

The Ampulla Muscle

The difference in sarcomere length (or A band length) of different fibres of the ampulla muscle raises the question of what functions do these different fibres serve. Conclusions reached from the work on crustacean muscles indicate that there is a correlation between the rate of contraction (and relaxation) and sarcomere length (Hoyle and McNeill, 1968a; Jahromi and Atwood, 1967, 1969a, 1971). Slow fibres have long sarcomeres while fast fibres have short sarcomeres. However "fast" and "slow" are relative terms (Hoyle and

and McNeill, 1968b), and depend on the particular anatomical location of the set of fibres as well as the species of animal. The correlation between sarcomere length and speed of contraction is not so well supported by insect muscles. Jahromi and Atwood (1969b), working on coxal muscles of Periplaneta, present extensive data showing that the contraction rate is dependent on sarcomere length and independent of other muscle parameters. Cochrane et al., (1969), came to a different conclusion following their investigation of leg muscles in Schistocerca. They found that the sarcomere length of phasic fibres in the extensor tibiae were much greater than that of tonic fibres of the same muscle. The structural feature which they could correlate with contraction rate was the amount of SR. The phasic fibres had almost three times the amount of SR compared with the tonic fibres. Huddart and Oates (1970), compared the prothoracic flexor tibialis of Carausius and the metathoracic extensor tibialis of Locusta. They concluded that the rates of contraction and relaxation of these two different muscles was dependent on the degree of development of the SR and the density of diads. They did not consider the fact that these muscles may contain fast and slow fibres and treated the problem from whole muscles.

From these data, and the lack of electrophysiological information, it is difficult to assign a physiological function to the different types of muscle fibres in the ampulla muscle.

The only point that can be claimed is that there is a good possibility that the ampulla muscle contains physiologically different fibres.

The function of Z tubules is not understood. They occur in some crustacean muscle fibres (e.g. Atwood, 1971), and some insect fibres (Hagopian and Spiro, 1967; Walcott and Burrows, 1969; Jahromi and Atwood, 1969b; Mill and Lowe, 1971). In insects, these Z tubules sometimes give rise to longitudinal elements which form diadic contacts with the SR (Walcott and Burrows, 1969; Hagopian and Spiro, 1967). In these cases, there is a dense layer, or hemidesmosome, against the Z tubule in line with the Z band. This structure is the same as that found in the ampulla muscle. The hemidesmosome at the Z band could be a mechanism whereby the myofilaments and sarcoplasmic invaginations are held together during contraction (Hagopian and Spiro, 1967).

The sarcolemmal invaginations at the A band level of the short sarcomere fibres of the ampulla muscle do not appear to have been reported previously. These square-sectioned invaginations must fulfil a role similar to conventional T-tubules since diads are formed between them and the SR.

Numerous mitochondria and abundant glycogen appear to be characteristic of muscles which are able to maintain

long contractions of high tension and of muscles which undergo rapid rhythmic contractions (Hoyle and McNeill, 1968a; Smith, 1968). The ampulla muscle has a large volume of mitochondria at the I-A overlap zone, but a lesser volume elsewhere. This arrangement of mitochondria is a combination of that found in flight muscle (Smith, 1968), and other skeletal muscle (Hagopian, 1966; Smith, 1966). This abundance of mitochondria and extensive distribution of glycogen would indicate that the ampulla muscle peforms a large amount of work during its rhythmic contractions.

The attachment of the muscle fibres to the ampulla wall have some of the structural features of muscle-hypodermis junctions (Shafiq, 1963; Lai-Fook, 1967; Caveney, 1969; Smith et al., 1969), and some features of muscle attachment to glandular structures (Smith et al.,1969). In muscle-hypodermis junctions, the adjacent plasma membranes are interdigitated and have sheet-like desmosomes connecting the two cells. The hypodermal cell has numerous close-packed microtubules which run from the apex to the base of the cell. The microtubules are parallel to the myofilaments. The attachment of the ampulla muscle to the wall has some of these features. The terminal sarcolemma possesses the characteristic hemidesmosomes. When there is a wall cell adjacent the muscle terminal, there is a full desmosome formed between them. The wall cell has

microtubules, but these are not as abundant as in the case of the muscle-hypodermis junction. A feature of the wall attachment cells is the development of a fibrous component along with the microtubules. This does not appear to be the case in other reported instances.

The majority of the muscle terminal zones abuts extracellular connective tissue. In these zones, the mode of attachment appears to be directly onto the connective tissue by hemidesmosomes. A fairly similar case is found in the venom gland of the black widow spider, Latrodectus mactans (Smith et al., 1969). In this case, the muscle is anchored to the extracellular connective tissue layer.

The degree of development of the hypodermal "tendon" cell onto which the muscle is attached is probably dependent upon the mechanical tension which is produced by the muscle. Hence, for skeletal muscles, a strong linkage is required. For deformable structures, such as the venom gland and the ampulla, high tensions are probably not developed and a "weaker" attachment is sufficient to fulful the function of these structures.

Neuromuscular synapses containing dense granules have been reported in numerous invertebrate animals. For instance, in the heart (Normann, 1965; Johnson, 1966a; Miller and Thomson, 1968; Komuro, 1970; and this work - see chapter on segmental blood vessels), in abdominal muscles

(Osborne et al., 1971; Maddrell, 1967), in leg muscles (Atwood et al., 1971), and in visceral muscles (Smith, 1968; Atwood et al., 1971). The occurrence of similar granule containing nerve endings in the ampulla muscle appear to fall into two groups. There are those which form neuromuscular junctions and those that form apparent release sites for NSM on the periphery of the muscle. The granules in the neuromuscular synapses may contain a transmitter substance or, alternatively, some kind of trophic material essential for neuromuscular integrity as suggested by Atwood et al., (1971), or perhaps a substance which affects the excitation threshold of the muscle (Osborne et al., 1971). The morphological distinction between neurosecretory granules and other types of granules (transmitters etc.) is difficult. However, the axons on the periphery of the ampulla muscle are most probably neurosecretory since they have no obvious close contact with the muscle cells. The axons forming the neuromuscular junctions possibly contain a granule-bound transmitter.

Herring Bodies

Herring (1908) described "masses of hyaline or granular character" in the neurohypophysis of the dog and cat. These structures are now called Herring bodies. From light microscopic studies, Herring bodies were considered to be axonal swellings filled with neurosecretory material (Gabe, 1966). More recent electron microscopic investiga-

tions of the neurohypophysis has shown that the terminal Herring bodies contain a variety of other organelles as well as neurosecretory granules, such as microtubules, numerous mitochondria, dense bodies, multivesicular bodies, tubular formations and multilaminar bodies (Bodian, 1963, 1966; Zambrano and De Robertis, 1967, 1968; Wittkowski, 1967, 1968a, 1968b; Bergland and Torack, 1969; Dellmann and Rodriquez, 1970; Polenov and Garlov, 1971). Herring bodies have been postulated to undergo cyclical degeneration and regeneration (Dellmann and Rodriquez, 1970; Polenov and Garlov, 1971). Degeneration and axon regeneration induced by nerve sectioning show similar accumulations in the severed nerve stumps to those found in Herring bodies (Dellmann and Owsley, 1969; Zambrano and De Robertis, 1968; Zelena et al., 1968; Rodriguez Echandia et al., 1970; Rodriquez and Dellmann, 1970a). Lysosomes are a consistent features of normal axon endings of neurosecretory neurons of the rat posterior pituitary (Whitaker et al., 1970), so the potential for degeneration is most probably present in all axon terminals.

The Herring bodies found in the ampulla wall are very similar to those showing signs of degeneration, exemplified by cattle (Dellmann and Rodriguez, 1970) and sturgeons (Polenov and Garlov, 1971). This appears to be the first report of such Herring bodies in insects. Herring bodies of Periplaneta in the first category are similar to those

showing early indications of degeneration (viz. numerous, closely packed mitochondria in the centre of the Herring body, and a few dense bodies). The second category is like those which are almost completely degenerated (viz. many multilaminar bodies). No cases were found in Periplaneta which corresponded to the regenerative phase which has large tubular formations. These tubular formations bud off dense granules, purported to be neurosecretory granules (Dellmann and Rodriguez, 1970). The only site where more than normal amounts of tubular material (probably smooth endoplasmic reticulum) was in axons running parallel to the ampulla muscle. Similarly, no cases were found which could be compared to "macro-apocrine" secretion (Polenov and Garlov, 1971) or "neuro-apocrine" secretion (Bodian, 1966). In these cases the degenerated material is extruded from the axon by breakdown of the plasmalemma and supposedly constituting a mode of hormone release. The lysosomes would, in all probability, break down the hormones as well as other intercellular constituents. This is supported by Rodriguez and Dellmann, 1970b), who found that the hormone content of Herring bodies decrease as degeneration proceeds.

Even though regeneration stages were not found, it does not preclude their existence. Judging from the speed at which degeneration and the commencement of regeneration occurs in transected axons (ca.18 hours, Rodriguez and Dellmann, 1970a), it is possible that the cases found were

only partly through a cycle. In only a few cases reported, has any indication been given on the duration of the degeneration/regeneration cycle of Herring bodies. Yakovleva (1966, quoted in Polenov and Garlov, 1971), reported that Herring bodies only occur in older sturgeons (3 to 5 years old). Other authors (quoted in Polenov and Garlov, 1971) also report the formation of Herring bodies in older animals. Pilgrim (1970) found a strong increase in the number of Herring bodies in old rats (15-20 months). He considered the dense bodies and multilaminar bodies to be distal accumulations of structures produced in the perikarya. However, these organelles are rarely found in the course of axons. Also, axoplasmic flow can occur in both directions (Dahlström, 1971). Whatever the cause of Herring body degenerative features, the effect is either a temporary or permanent interruption to neurosecretory material release.

The co-existence of large degenerating Herring bodies and smaller "normal" neurosecretory axon terminals in the ampulla wall, both of which contain morphologically similar NS granules, would suggest that a general pathological condition does not exist. The Herring bodies are essentially pre-terminal (in the sense of release sites), since they have a glial investment and no small vesicles. The glial envelope may be a reaction which occurs simultaneously with the onset of degeneration. If it is

assumed that these Herring bodies are derived from once actively secreting terminals, then the increase in size can probably be accounted for by an accumulation of NS granules which are moving distally (mostly) down the axon. The mechanism which induces Herring body degeneration is unknown, but may be involved with ageing. Accumulations of lipofuscin granules in perikarya is a well known effect of ageing. Lipofuscin granules are though to be the final stage of lysosomal digestion of organelles (Peters et al., 1970).

Apart from the cases cited for vertebrates, there is no comparable report in insects (or other invertebrates) of degeneration in Herring bodies. It would be expected that if Herring body degeneration (short term or long term) is an integral part of NS terminals, then it would be present in other neurohaemal organs such as the corpora cardiaca and perisympathetic organs. In no cases reported, has anything like degeneration on a large scale been found.

Many explanations for this occurrence can be presented, but there is not a great deal of evidence to substantiate the hypotheses. Long term formation, such as ageing, has already been discussed. Short term formation requires some qualification. The degeneration may be a reaction to overproduction of NSM. Over production could be a mechanism by which an animal ensures that there is a large stores of NSM present in the terminals which can be

drawn upon in times of physiological crisis. To remove excess NSM, the terminal Herring bodies undergo degeneration, and then regenerate to form a new functional terminal. There does, however, appear to be some feed-back mechanism which regulates output from the perikarya in response to demand. For example, the NS cells in the preoptic nucleus of the guinea pig incorporate 2 to 5 times more radioactivity into vasopressin after 4 days water deprivation (Sachs, 1970). It would appear as if there is a fairly slow reaction in the rate of output. Similarly, there is probably a slow reduction in output, which could lead to an excess of NSM at the terminals, provided axoplasmic transport is continuous at a steady rate. However, Jasinski et al., (1967) found that the rate of depletion of stainable NSM was much faster in the preoptico-hypophysial system of goldfish (in the order of minutes). The perikarya were the first to show signs of depletion followed by the axon tracts and finally, but rarely, the terminal regions. Herring bodies throughout the axon tracts possibly represent local accumulations of NS granules which are in transit, and not degenerating zones. However, the terminal Herring bodies may perhaps represent degeneration zones since they are rarely depleted by acute stimulation. After stimulation, the NSM builds up from the terminal end of the axon and the perikarya are the last to show signs of accumulation. The whole neuron appears to be able to store NSM.

The rate of synthesis of NSM is increased during

a demand period. When the demand is reduced, secretion slows rapidly (electrically controlled), but the synthetic processes would require a longer time to slow down. The excess NSM produced could be transported down the axons and accumulated in the terminals and degeneration follows. But why can't the NSM be stored indefinitely? This would appear to be an efficient way of ensuring a ready pool of NSM and would also conserve nutrients and energy used in synthesis.

From the available data, it is difficult to draw any definite conclusions. Herring bodies which show degenerative features are widespread, but their physiological function has yet to be fully elucidated.

Ampulla Wall

The cellular layer of the ampulla wall has several features in common with the inner layer of cells of the convoluted part of the vessel leading from it. The cell junction specializations have their counterparts in the vessel wall as do the bundles of microtubules on the lumenal side. The functional significance of these structures will be treated in the discussion of the vessel wall. It is sufficient to say that the ampulla wall constitutes a "water tight" barrier for most of its expanse.

The other interesting characteristic of the wall is the middle extracellular layer. It would seem as if this layer imparts elasticity to the wall. On contraction

of the ampulla muscle, the lumen is filled with haemolymph by stretching the wall. Relaxation of the muscle releases the wall from its expanded state and the ampulla springs back to its relaxed position, expelling the haemolymph through the ampulla vessel. The greatly developed middle layer would suggest that it is responsible for the elasticity. Also, there are no signs of any muscle cells in the ampulla wall which could account for the elastic properties. The random orientation of fibre bundles indicates that tension is not developed in one direction only and would accommodate stretching in all directions.

Jones (1964) reports that the ampullae will continue pumping even after the muscle is cut. This cannot be accounted for, considering the structure of the wall. Although he does not mention any technical details, this conundrum may be explained. If the ampulla muscle is exposed by removal of part of the frons and then cut, then the anterior aorta would still remain in continuity with the ampulla. Contraction of this muscle could possibly rhythmically move the ampulla. Any mechanism involving rhythmic pulsation of the ampulla itself would appear to be impossible considering its structure.

Jones (1964) also quotes some other data on the functioning of the pulsatile organ. The rate of contraction varies from 26 to 96 per minute. The pulsations are not synchronous with those of the dorsal vessel. This would

indicate that the controlling mechanisms of the two structures are different. The nervus connectivus runs from the frontal ganglion to the protocerebrum (Willey, 1961), apparently passing through the ampulla muscle. Pawlowa's (1895) original description states that the muscle is innervated from both the frontal ganglion and the protocerebrum. Clearly, the nervus connectivus passes through the muscle, but it is unknown whether or not it supplies axons to the muscle. The other possible source of innervation is from the nerves running along the anterior aorta. These nerves most probably originate from the retrocerebral complex and may serve as neurohaemal release areas as well as supplying the ampulla muscle. Also, since the neuromuscular junctions contain dense granules similar to some of those found in the anterior aorta nerves, it would appear as if this is a source of innervation for the ampulla muscle. The retrocerebral complex also gives rise to a posteriorly directed aortal nerve (Willey, 1961), which may have some control over heart rate. However, the heart receives extensive segmental innervation from the ventral nerve cord as well as intrinsic ganglion cells in the lateral cardiac nerve (Alexandrowicz, 1926). The heart appears to be controlled from these latter sources (Miller, 1968; Miller and Usherwood, 1971). At this stage, the origin of the controlling mechanism of the ampulla muscle is uncertain.

The Antennal Vessel

The convoluted portion of the vessel appears to be specialized for purposes other than supplying haemolymph to the antenna. The length of the vessel between the ampulla and the base of the antenna is more than sufficient to accommodate antennal movement. The extra length is probably related to the specialization of the cells forming the walls. The convoluted portion may be of such dimensions as to allow some modification of the haemolymph before it enters the antenna. The time required for this process would be dependent upon the rate of flow of haemolymph, the rate of the process, as well as the physical dimensions of the vessel. The convolutions may ensure a sufficient time for the blood to be processed fully before reaching the antenna.

This idea is supported by the form of the wall cells in the head capsule and in the antenna. In the latter, the walls are thin and although they have some structural features in common with the basal portion, they have no special features which would implicate them as serving any other function than conducting haemolymph. The wall cells in the head capsule are markedly specialized, and possess many features in common with other epithelial cells which actively transport fluids (see Physiological Society Symposium on Comparative Aspects of Transport of Hypotonic, Isotonic and Hypertonic Solutions by Epithelial Membranes).

The main structural features of fluid transporting epithelia are of a general type, no matter what the species of animal investigated. The epithelial cells have some sort of dead-end channel. This channel may be an intercellular space which is sealed at one end by "tight" junctions and/or septate desmosomes. Alternatively, the channels may be basal infoldings or intracellular canaliculi. The channels open in the direction of fluid flow. Usually, the opening is restricted so that the channels are almost isolated from other extracellular spaces. The cell surface opposite the channels usually has some type of microvillus structure. There are abundant mitochondria which supply energy for the active transport process. These may be distributed towards one end of the cell or be associated with the apical and/or basal plasmalemma, and sometimes with lateral cell membranes.

The geometry of the dead-end channels is important in determining the osmolarity of the fluid transported. Briefly, for solute transport and water transport coupling, standing gradients of osmolarity are developed in the channels by active transport. If the channels are long and narrow, then isoosmotic transport can be achieved. However, if the channels are short and wide, then hyperosmotic transport can occur. Hypo-osmotic transport requires an extension of this arrangement to facilitate ion recycling in a secondary compartment. This last type of transport occurs in the

insect rectum and is the only case where local osmotic effects have been demonstrated so far (Wall, et al., 1970).

The structure of the convoluted portion of the vessel has many similarities with other epithelia which are involved in fluid transport. On the lumenal side (apical), the inner layer of cells have microvillus-like projections. However, these projections are different from other epithelia in that they possess many microtubules. Basally, the cells have extensive extracellular spaces which only rarely communicate with the general blood sinus through small gaps between the outer cells. The extracellular spaces are filled with connective tissue (basement lamella and collagen fibres). This is fairly unusual, but a somewhat similar situation is found in the rectal papillae of Calliphora (Gupta and Berridge, 1966). The adjacent cell junctions are specialized. Tight junctions, septate junctions, and "ordinary" desmosomes occur between adjacent cells of the inner layer. The distribution of mitochondria is polarized within the cell. They are accumulated towards the apical surface, presumably where the active transport processes would be occurring.

These structural modifications are not present in the antennal portion of the vessel. If the blood is to be modified for the antennal environment, then this is only to be expected since further modification within the antenna would be superfluous and unnecessary.

Some of the structural features of the convoluted portion of the antennal vessel do not appear to fall into the general pattern for transporting epithelia and deserve further comment. The masses of microtubules in the apical projections are probably the most striking deviation from what might be expected. Microtubules are found in most, if not all, cell types. They have had numerous functions assigned to them. Amongst these functions are a cyto-skeletal role (e.g. mitotic spindle, Ledbetter and Porter, 1963), and involvement with transport down axons (e.g. Smith, D.S. 1971), cytoplasmic movement in ovarioles (Macgregor and Stebbings, 1970), pigment migration in melanophores (Bikle et al., 1966), as an intermediate in the attachment of arthropod muscle to the cuticle (e.g. Smith et al., 1969) as well as a mediator of water transport (e.g. Witkus et al., 1969). Microtubules supposedly have the same structure, irrespective of their occurrence. If this is so, then their versatility of function is amazing. Perhaps they are associated with other ill-defined structures which are more variable in nature and the combination is the determining parameter which allows particular functions. (e.g. acting filaments are present in sperm tails and meiotic spindles (Behnke et al., 1971), and mitotic spindles (Gawadi, 1971)).

Perhaps microtubules serve as a means by which water and small ions can be transported through the cytoplasm. This hypothesis was put forward by Lane and Treherne (1970a). They found that lanthanum staining made the usually clear halo

around the microtubules, as well as the central core, to become opaque. It was suggested that the lanthanum reveals acid mucopolysaccharides or other anionic molecules. These substances could serve as a matrix for ion and water transport. This interpretation would fit into the proposed function of the antennal vessel cells.

The occurrence of numerous dense bodies of variable size is difficult to explain. Usually, dense bodies are primary lysosomes which contain hydrolytic enzymes (Novikoff, 1967). They seem to be too abundant to be involved in cellular "wear and tear" only. Also, their association with the microtubules makes interpretation of function difficult.

The vacuolar nature of much of the rough ER and dilated form of the nuclear envelope may be due to osmotic stress during primary fixation of the tissue. A similar type of swelling has been demonstrated in isolated flounder nephrons by either osmotic shock or inhibiting the sodium transport process (Trump and Bulger, 1971). In the antennal vessel, it is probably a fixation artifact. No attempts were made to modify the osmolarity of the fixative, so this conclusion must remain tentative.

The occurrence of annulate lamellae is widespread in animal cells (see reviews by Kessel, 1968 and Wischnitzer, 1970). Kessel (1968) came to the conclusion that they are transitory structures which occur in young, growing and

differentiating cells. Wischnitzer (1970) goes further, and includes cells which have a high metabolic activity. Most annulate lamellae are derived from the nuclear envelope which they resemble fairly closely. Their function, however, is not clear. Perhaps they are involved in transport of nuclear material into the cytoplasm (Kessel, 1968). Recently, annulate lamellae have been found in insect prothoracic glands (Cassier and Fain-Maurel, 1970), and rectal pad cells (Oschman and Wall, 1969). They also occur in muscle cells of excurrent ostia (see chapter in this work), as well as in the antennal vessel cells. In these cases, there is a continuity between the annulate lamellae and rough ER. Perhaps this indicates some role in protein metabolism of cells which are metabolically very active.

The specialized cell junctions found in most epithelia are postulated to serve several functions. Desmosomes (macula adhaerens and zonula adhaerens types) probably serve as intercellular attachment devices (Farquhar and Palade, 1963). Septate desmosomes, which are common in invertebrate epithelia, usually form a seal around cells and prevent diffusion of extracellular solutes between the cells. They have been implicated in intracellular electrotonic coupling (Loewenstein and Kanno, 1964; Wiener, Spiro and Loewenstein, 1964), but this has been challenged recently (Hudspeth and Revel, 1971). These latter workers suggest that the "tight" junctions are the pathways of low-resistance coupling. (Many

"tight" junctions have been shown to be gap junctions: there is a narrow gap of ca. 3nm between adjacent membranes and not complete fusion of the outer leaflets of the unit membranes). They also make a warning in the cases where both septate desmosomes and gap junction occur together. In these cases, it cannot be decided which type of junction is responsible for electrotonic coupling, but since gap junctions only have been found in many coupled cells (mainly vertebrate examples), it is most probable that they are responsible.

Low-resistance junctions allow the passage of molecules between cells (e.g. Sheridan, 1971; Lane and Treherne, 1970b). The implications of this function is that adjacent cells may form a diffusion pathway for ions or molecules. This might allow the tissue to react in a uniform manner to stimuli from nerves and/or hormones. One such molecule may be 3'-5' cyclic adenosine monophosphate (cyclic AMP). This substance is a "second messenger" in many hormonal reactions (Sheridan, 1971). In glial tissue, gap junctions have been implicated in the diffusion pathway of ions between the haemolymph and the neuronal environment (Lane and Treherne, 1970b). In this way, ionic movement may be controlled quite critically. This latter fact may be important in the function of the antennal vessel wall. Also, the inner and outer layers of cells have "tight" junctions between them at many points. This may allow a

buffering effect by the outer cells on the movement of ions across the walls. Also, the extracellular spaces between the two layers of cells may act as a cation reservoir since it contains connective tissue (mucopolysaccharides and collagen) which forms an anionic matrix (see Treherne and Moreton 1970).

The stability of ionic concentration is important in the function of excitable tissues (see Narahashi, 1963). The ionic concentrations in the haemolymph of insects is very variable and is related to the diet, age and degree of hydration of the animal (Pichon, 1970). The neuronal environment in the central nervous system is controlled by the perineurium/glial cell system (Treherne and Moreton, 1970). In this case, the "insulating" system is well developed and the internal environment of the CNS is relatively constant compared with the external haemolymph. In the antenna, the glial sheath does not necessarily cover all nervous tissue and, in some cases, the neurons are exposed to the haemolymph (e.g. Slifer, 1967; Zacharuk et al., 1971). In the cases where there is a glial sheath, such as in the case in hand, the glia is not well developed. Also, it does not possess the morphological attributes of tissue which controls and/or water transport. It could be expected that the glial tissue does not closely control the ionic environment of the sensory neurons.

The antennae of Periplaneta americana have been shown to have well developed chemoreceptors and hygroreceptors

(Guthrie and Tindall, 1968). For their function, it would be expected that very constant ionic conditions would be required to give an acceptable signal/noise ratio. Pichon (1970) found that the ionic content of haemolymph taken from the antennae was quite different to that from the dorsal vessel as well as other regions of the body.

IONIC COMPOSITION OF HAEMOLYMPH
(mM/Kg haemolymph)

(from Pichon, 1970)

	Na^+	K^+	Ca^{++}
Antenna	165.2 ± 6.4	13.8 ± 1.2	4.25 ± 0.36
Dorsal Vessel	147.6 ± 2.3	18.8 ± 3.0	3.8 ± 0.13
	$P = 0.01$	$0.1 < P < 0.2$	

The only access route to the antennae for the haemolymph is by way of the ampulla/antennal vessel system. This system is ideally placed to have some sort of control over the haemolymph entering the antennae. Also, the structure of the convoluted portion in the head capsule is very suggestive of an epithelium involved in water and/or ion transport. This system could smooth out fluctuations in composition of the haemolymph before it enters the antenna.

It may even give a constant composition irrespective of the body haemolymph fluctuations.

The control of this system may be mediated by the neurosecretory material found in axon terminals in the ampulla. Haemolymph passes to the antenna only from the ampulla and hence any released neurosecretory material would go directly to the antennal vessel. Other systems concerned with ion and water transport in insects, such as the Malpighian tubules and the rectum, are controlled by neurosecretory substances (e.g. Maddrell, 1966; Mordue, 1969; Pilcher, 1970; Wall, 1967). A similar control system may exist in the antennal system. The transducer which measures ionic composition has not been identified. It probably synapses with neurosecretory neurons in the brain or corpora cardiaca, since this seems to be the origin of the neurosecretory terminals in the ampulla.

GENERAL DISCUSSION

Only in recent years has the extensive distribution of neurosecretory (NS) cells and axons within the insect body been realized. Certainly, the occurrence of all NS neurons is not known and this point is adequately shown by the examples presented in this thesis. Even in the extensively studied insect Periplaneta americana, there are many previously unsuspected sites where NS cells and axons occur.

The acridine orange vital staining technique is one method by which NS material may be identified. This technique is useful for various invertebrates since the size of tissue samples in these animals is generally small. One disadvantage of the technique is that the preparations are not permanent and a photographic record is required. However, this is outweighed by the speed and specificity of the method.

The NS system in the optic lobe is entirely new and no similar system has previously been described from other insects. The system is characterized by NS material which will not stain with paraldehyde fuchsin but remains acidophilic after permanganate oxidation. The NS material is stainable with Heidenhain's azan and Mallory's triple stain. The NS cells appear to be a homogeneous group at both the light and electron microscope level although there are slight differences in stainability of individual cells. With such a group it is possible to correlate the staining

characteristics (i.e. "Gomori-negative") with electron microscope appearance. In this case, C type NS cells correspond to electron-dense granules. From the histochemistry, the NS material appears to be a peptide.

The unipolar form of the optic lobe NS cells is a feature shared with other NS cells in insects. Synapses on processes of these cells were found. The presynaptic element contains small electron-dense granules which could contain a biogenic amine. The actual process within the NS cells which these synapsing axons control can only be speculated upon. However, it does suggest that the NS cells are at least second order in some process. The afferent stimulation may well come by way of the small-granule-containing axons, but the location of their perikarya and their receptor sites were not found.

A significant diurnal variation was found in the size of the optic lobe NS cells and their nuclei. The peaks in size corresponded to changes in the light regime. The transition from light to dark corresponded with the largest peak in size and also with the most rapid change in size. The animals were also most active during this period. The relationship between NS cell activity and circadian rhythms was tested experimentally but the results were not conclusive and it was not possible on the evidence available to say whether or not there is a direct causal relation between the circadian activity rhythm of the insects

and NS cell size.

There is a diurnal variation in the number of release sites in NS axons of the lateral cardiac nerve and of the segmental blood vessels. The number at night is greatly in excess of the number during the day. This correlated with the nocturnal habits of P. americana. The released NS material probably acts as a metabolic hormone since material released into the lumen of the vessel would be carried directly to the body cavity. The diurnal variation in numbers of release sites indicates that formation of release sites and probably of synaptoids is transitory, and that they are not more permanent structures like true synapses.

There is a certain amount of evidence that NS material is released by exocytosis. The finding of increased numbers of release sites at night increases the probability of finding evidence which will throw more light on this process. Fixation of tissue at the time when this animal (and perhaps other animals) is most active would appear to be most expedient for investigation of the release process. This approach may be helpful in resolving the dilemma of the release mechanism; whether it is by exocytosis and retrieval of the axolemma by formation of small vesicles, or by fragmentation of neurosecretory granules and diffusion, or by both of these methods within the one animal.

Recently published evidence from studies of other insects suggests that the first alternative is most probably the correct one (e.g. Smith, U. 1970).

The valves of the segmental blood vessels are innervated by granule-containing axons. It would appear from the morphology of these neuromuscular synapses that the granules contain a transmitter substance, perhaps a biogenic amine. In this case, the granular material should be called a neurohumor rather than a neurohormone (Scharrer, 1969). Similarly, axons containing small granules which synapse with the optic lobe NS cells would appear to be involved in impulse transmission and the material contained in the granules should be called a neurohumor. The division between neurohumor and neurohormone becomes blurred in the case of granule-containing axons in the ampulla muscle and in the degenerating muscles of the abdomen. In some cases, there is apparent release of material close to the muscle but there are no synapses. On morphological grounds, it is hard to classify such axons. They have been called neurosecretory axons in this thesis.

Many peripheral nerves in the abdomen carry at least some NS axons. The unpaired median nerves possess the well known perisympathetic neurohaemal organs near the ventral nerve cord. The lateral branches of this nerve also carry many NS axons which appear to form a lateral neuro-

haemal organ as well as supply the lateral cardiac nerves with NS material. This wide distribution of NS axons raises the question of how many functions are controlled by this system and whether or not there is any simultaneous control of different functions. For example, is there simultaneous or independent release of similar or different NS substances in the perisympathetic neurohaemal organs, lateral neurohaemal organs and cardiac neurohaemal organs? Or is the release of one type of substance dependent on the release of some other type? What is the stimulus which invokes release? The structure of this system points to many important functional questions.

NS cells outside the central nervous system do not appear to be of general occurrence, or perhaps they have not been looked for on a systematic manner. The presence of the two NS cells on each link nerve raises questions of their origin, control and function. Since these cells are essentially isolated from other neurons, they may form a convenient preparation for the investigation of the neurophysiology of NS cells.

The sets of muscles in the lateral region of the abdominal segments which degenerate in the imago are an example of internal metamorphosis which is not generally recognised in hemimetabolous insects. The nature of triggering mechanism to induce degeneration is not known, and the literature on the subject is full of conjecture.

Perhaps the NS axons associated with the muscles are involved, but it is also possible that the muscles and subsequently the stroma sheaths may only be a convenient structure to support extensions of the lateral neurohaemal organs.

The pulsatile muscle of the antennal vascular system is composed of two types of fibres which have different sarcomere length. This morphological difference may well reflect a physiological difference between the two types of fibre; a situation which is often found in arthropod muscles. Again, there are granule-containing axons associated with this muscle which have similarities with axons of the heart and segmental blood vessel valves. This appears to be a general feature of visceral muscle which performs a rhythmic function.

The neurohaemal organs in the walls of the ampullae are of a different type from those found in other parts of the insect nervous system. The formation of Herring bodies, some of which show signs of degeneration, is characteristic of the antennal neurohaemal organs. The contents, size and number of the Herring bodies may be related to the physiological state of the animal or perhaps to its age. From its anatomical location, this neurohaemal organ probably releases material which affects some aspect of antennal function. Whether or not it is involved with the convoluted portion of the antennal vessel, which appears to be an ionic

regulator, or with receptor function is not known. The whole pulsatile organ and its associated parts appear to constitute an important component of antennal function.

The neurosecretory systems investigated during the work for this thesis indicate that there are many unexplored facets of insect structure, and that the physiology of these systems is in an even less advanced state.

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Vital Staining of Neurosecretory Material with Acridine Orange in the Insect, *Periplaneta americana*

Neurosecretory materials vary in their chemical composition between different neurosecretory cells and no one staining technique has been found to stain all types of neurosecretory material[1]. In this paper it is shown that acridine orange can be used as a vital stain for all neurosecretory materials.

Vital staining of cells with acridine orange produces an orthochromatic green fluorescence in nuclei and sometimes a metachromatic red fluorescence in cytoplasmic granules. It is now generally accepted that green nuclear fluorescence is due to nucleic acids and that the red cytoplasmic granules are lysosomes[2–8], however, see AUSTIN and BISHOP[9] for an alternative interpretation. As well as staining with acridine orange, lysosomes show acid phosphatase activity amongst other hydrolytic enzymes[2–5].

Materials and methods. Adult and nymphal forms of the cockroach, *Periplaneta americana* from laboratory colonies were used in this study. The following stains were obtained from Chroma-Gesellschaft; acridine orange, acridine yellow, coriphosphine, acriflavine, phosphin 3R, euchrysin 3RX, and euchrysin 2G. All are acridine derivatives. They were dissolved in 0.9% NaCl at a concentration of 0.1 mg/ml.

Pieces of nervous tissue were dissected from the insect under saline and placed in a drop of stain solution on a microscope slide. The tissue was stained for 1 min and then moved to a drop of saline further along the slide. A coverslip was added and excess saline removed. The whole mount was then viewed with blue light from a Wild microscope fluorescence system.

For acid phosphatase localization, tissue was fixed in formol-calcium over-night. The Gömöri lead method and the simultaneous coupling azo dye method using naphthol AS-TR and hexazotized pararosaniline were used[10]. Incubations without substrate were used as controls.

The stains were analyzed by thin-layer chromatography using Kieselgel and *n*-butanol:ammonia:ethanol:water (16:0.15:5:5) as developing solvent[11]. The chromatograms were viewed with long-wave UV-light.

Results. Differentiation of neurosecretory cells and 'ordinary' nerve cells was obtained with acridine orange, euchrysine 3RX, and coriphosphine, however the latter showed only weak differentiating ability. Negative results were obtained with the other stains. Neurosecretory material appeared as red fluorescing granules while the cytoplasm of 'ordinary' nerve cells and axons was a uniform weakly fluorescent green. Nuclei of glial and nervous tissue showed a strong green fluorescence with distinct nucleoli. The cytoplasm of glial cells was not stained. The red fluorescence was not stable under continuous irradiation and faded after 5 min. The nuclear

staining retained its colour for up to 15 min before turning yellow. After 20 min irradiation the tissue took on a general red tint which has been taken as indicative of cell death[6].

Suitable tissue for comparison of neurosecretory and 'ordinary' axons were the lateral cardiac nerves in which the 2 types of axons are fairly well partitioned into 2 bundles[12]. After staining, the more lateral bundle of neurosecretory axons was found to contain dense masses of red granules while the 'ordinary' axons were a weakly fluorescent green. The neurosecretory axons of the lateral cardiac nerves cannot be stained by the classical neurosecretory stains, but at the ultrastructural level they have been shown to contain elementary neurosecretory granules[12].

In the head, neurosecretory cells in the pars intercerebralis could be distinguished from neighbouring cells because their cytoplasm was almost completely filled with red granules while 'ordinary' nerve cells contained only a few large red granules which were probably lysosomes. Also, the axons of these cells contained considerable numbers of small red granules. The nerves to the corpus cardiacum showed red granules in some axons and only weak green general staining in others. The corpus cardiacum itself possessed scattered red granular accumulations among the intrinsic cells whose nuclei stained green. This distribution of neurosecretory material agrees with electron microscope studies made by SCHARRER[13]. Nerves leading to, around, through, and away from the corpus allatum all exhibit large accumulations of red granules. Those axons passing through the corpus allatum often had a varicose arrangement of red granules. Again, this distribution agrees with electron microscope studies made by SCHARRER[14]. Also, the median nerves of the ventral nerve cord exhibit red granules in some of the axons proximal and distal to the neurohaemal organs[15, 16].

Since it was thought that neurosecretory material in other animals may give a similar reaction with acridine orange, the following representative systems which contain neurosecretory material were investigated; the eyestalk and pericardial organ of the crab, *Paragrapsus gaimardii*, the retrocerebral complex of *Calliphora stygia*, the heart of *Helix aspersa*, and the cerebral ganglion of the earthworm, *Megascolides polynephricus*. In each case neurosecretory cells and/or axons could be distinguished from 'ordinary' nerve cells and axons.

Acridine orange and euchrysine 3RX gave similar chromatograms which showed 6 (possibly 7) components with the slowest moving spot being the major constituent. The chromatogram of coriphosphine showed a fairly strong spot with an Rf value and fluorescent colour similar to the major component of acridine orange, but the other spots were all different in Rf value and/or fluorescent colour. Acid phosphatase was not localized in either type of axon in the lateral cardiac nerves by either of the methods used.

Unstained whole mounts of lateral cardiac nerves were viewed under dark field illumination. Neurosecretory material exhibited its characteristic blue colour in the form of granules or dense accumulations. The distribution of neurosecretory axons revealed by this method was similar to the distribution shown by vital staining with acridine orange.

Discussion. The metachromatic red fluorescence of neurosecretory material when stained with acridine orange allows a rapid method for identifying neurosecretory cells and axons. All the known sites of neurosecretory material in the insect nervous system give positive results by this method whereas previously different staining techniques and electron microscopy have been necessary to show the presence of all types of neurosecretory material. A similar staining method[17] has been used on the nerves in the mesentery of the frog, *Rana temporaria* and the cerebral ganglion of *Lumbricus terrestris* and it was found that the nerve cell bodies and axons contain red granules. Some of the granules have a varicose arrangement in the axons. This, together with the positive reaction in the neurosecretory cells and axons of the other animals used in this study, would indicate that all neurosecretory granules have some common feature which makes them stain with acridine orange. Whether this a particular chemical compound or a particular type of membrane structure which combines with acridine orange is not known. However, the entities responsible possess a highly orientated array of negative binding sites which are spaced at a distance which allows interaction between the adjacent acridine orange molecules[11].

The absence of acid phosphatase in the neurosecretory axons would suggest that it is not the presence of lysosomes which produce the metachromatic staining, but that the neurosecretory material itself takes up this stain. Whether this similarity in staining is due to similar compounds or membranes is unresolved, but both lysosomes and neurosecretory granules contain biologically active substances in a latent form[1, 5].

The thin layer chromatographic analysis of the 3 stains which gave positive results indicate that acridine orange and euchrysine 3RX are the same, or very similar, products and that coriphosphine is contaminated with acridine orange. It is this which gives metachromatic staining of neurosecretory material[18].

Résumé. On expose une méthode rapide de la coloration vitale à l'acridine orange des produits de neurosécrétion dans le tissu nerveux des insectes. Irradiés avec la lumière bleue, tout les types connus de produits de neurosécrétions entrent en fluorescence en prenant une teinte métachromique rouge.

T. M. BEATTIE

University of Tasmania, Department of Zoology, Hobart (Tasmania 7001, Australia), 4 May 1970.

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HISTOLOGY, HISTOCHEMISTRY, AND ULTRASTRUCTURE OF NEUROSECRETORY CELLS IN THE OPTIC LOBE OF THE COCKROACH, *PERIPLANETA AMERICANA*

T. M. BEATTIE

Department of Zoology, University of Tasmania, Hobart, Tasmania, Australia

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Abstract—In the region of the distal optic chiasma of each optic lobe of *Periplaneta americana*, there is a group of about 120 monopolar neurosecretory cells. These cells do not stain with paraldehyde fuchsin but remain acidophilic after oxidation. They stain red or sometimes indigo with the azan technique. Histochemically, the neurosecretory material is positive for protein and the amino acids tryptophan and arginine but negative for 1, 2-glycols and strongly acidic groups. At the ultrastructural level, the cytoplasm of the cells contain many elementary neurosecretory granules 100 to 170 nm in dia. The cells also contain well-developed Golgi bodies and endoplasmic reticulum. The axons from these cells run toward the interior of the optic lobe. In this region, axons containing dense granules (mean diameter 70 nm) and synaptic vesicles synapse onto the axons from the neurosecretory cells. The neurosecretory axons then cross over to the anterior side of the optic lobe and run towards the brain. The function of these neurosecretory cells is unknown, but they may be involved with photoperiodically controlled activity rhythms.

INTRODUCTION

THE OCCURRENCE of neurosecretory neurones in the nervous system of insects is widespread, but the function of many of these neurones has not been elucidated (MADDRELL, 1967). Cytological evidence is, however, the usual starting point in any investigation of neurosecretory systems and serves as a basis for physiological studies. The staining properties of neurosecretory material have been shown to be diverse and not all types give a positive reaction with the basic stains, paraldehyde fuchsin and chromehaematoxylin, but may react with acid stains. Perhaps more indicative of neurosecretory cells is the production of elementary granules by the Golgi bodies in the perikaryon when the tissue is studied at the ultrastructural level.

The occurrence and distribution of neurosecretory cells in the brain of insects is well documented (GABE, 1966). In the case of *Periplaneta americana*, WILLEY (1961) has described groups of cells in the pars intercerebralis which give rise to the nervi corporis cardiaci I and II. Furthermore, KHAN and FRASER (1962) have described median, lateral, and ventral groups of neurosecretory cells containing

paraldehyde fuchsin–positive granules. Little work has been done on PAF-negative neurosecretory cells in *P. americana* although there has been several recent histochemical studies on this type of cell in other insects (RAMADE, 1969; RAABE and MONJO, 1970).

The ultrastructure of neurosecretory cells in the pars intercerebralis of *Blaberus craniifer* has been studied by WILLEY and CHAPMAN (1962). They found that the neurons contain granules up to 150 nm in dia, associated with the Golgi apparatus. Similarly, BERN *et al.* (1961) found electron-dense granules of about this size in the neurosecretory cells of the pars intercerebralis of *P. americana*. The cells in both these studies are most probably PAF-positive.

There has been no previous report of neurosecretory cells in the optic lobe of insects which is in contrast with the situation in crustaceans (GABE, 1966). In this present study the histology, histochemistry, and ultrastructure of a new group of neurosecretory cells in the optic lobe of *P. americana* is described.

MATERIALS AND METHODS

For light microscopy, whole brains with optic lobes were dissected from adults and larvae and fixed in Bouin's fluid. Sections of 6 μ were stained with paraldehyde fuchsin (EWEN, 1962), or with a modified azan technique (HUBSCHMAN, 1962). The following histochemical tests were carried out: mercury bromophenol blue for proteins (MAZIA *et al.*, 1953), PAS for 1,2-glycols, DMAB-nitrite for indol derivatives (ADAMS, 1957), diazo-safranin for 5-hydroxytryptamine and related substances (LILLIE *et al.*, 1953), the Liebman method for arginine (LIEBMAN, 1951), and the alcian blue/alcian yellow technique for acid groups (PEUTE and VAN DER KAMER, 1967).

For electron microscopy, optic lobes were fixed for 1 hr in 3% glutaraldehyde in 0·1 M phosphate buffer (pH 7·3) and post-fixed in 2% OsO_4 in buffer. Epon embedded tissue was sectioned and stained with uranyl acetate and lead citrate.

RESULTS

The first indication that there may be neurosecretory cells in the optic lobe of *P. americana* was the observation that two small patches of bluish tissue could be seen on the posterior aspect of the optic lobe when the head was dissected. This Tyndall effect, which is characteristic of neurosecretory material, prompted further investigation.

The neurosecretory cells form a flattened cone-shaped group on the posterior face of the optic lobe, immediately beneath the perineurium in the region of the chiasma between the lamina ganglionaris and the medulla terminalis (Fig. 1). These cells lie lateral and ventral to the main optic trachea at the point where it branches. There are about 120 monopolar cells in each optic lobe, and are usually ovoid to pyriform in shape. The cells measure about 16 μ through the major axis and 9 μ through the minor axis. Nuclei are round to ovoid and may measure 10 μ by 6 μ. The neurosecretory cells are larger than the surrounding neurons and possess a greater proportion of cytoplasm. Sometimes an axon hillock can be

seen and more rarely an axon can be identified. In these latter cases the axon runs towards the centre of the distal optic chiasma (Fig. 2).

The neurosecretory material (NSM) does not stain with paraldehyde fuchsin, but it does react with the light green component of the counterstain. With the azan technique, NSM is stained either red or sometimes indigo. Often the entire cytoplasm is filled with stainable material, but in other cases the NSM occurs as isolated patches in the cytoplasm. The histochemical tests showed that the NSM is positive for proteins, negative for 1,2-glycols, and contains no strong acidic groups which may have been derived from S—S or S—H groups. Also, there was only a slight reaction for weakly acidic groups. The DMAB-nitrite reaction was positive, indicating the presence of an indol derivative and this is most probably tryptophan since the diazo–safranin reaction was negative. Anginine is present in high concentrations in some of the cells whereas in others only a moderate amount of this basic amino acid is present. When viewed with phase contrast optics, the cells rich in arginine show very granular nuclei whereas the other neurosecretory cells have almost clear nuclei.

At the ultrastructural level, the neurosecretory cells contain masses of elementary granules (Figs. 3, 7). These are electron-dense and the majority have a diameter in the range 100 to 170 nm. The shape of the granules is spherical to ovoid. The number of granules in the neurosecretory cells seems to vary between individual animals. In some, the granule fill every available space; in other animals they are scattered more sparsely amongst the other cell organelles. Rough endoplasmic reticulum is usually fairly well developed and the cisternae often form concentric layers around the nucleus. There appears to be some correlation between the number of granules and the degree of organization of the endoplasmic reticulum: large numbers occurring with good organization and fewer numbers occurring with poor organization. Ribosomes stud the membranes of the endoplasmic reticulum but also occur free in the cytoplasm either singly or in groups. At the places where there is an accumulation of ribosomes on the endoplasmic reticulum, the material between the membranes is slightly more electron-dense. This may indicate a local concentration of protein at the site of synthesis by the ribosomes. Golgi bodies are well developed and show a marked polarization in their structure (Figs. 4 and 5). The cisternae contain electron-dense material and occasionally an almost complete elementary neurosecretory granule can be seen at the periphery of one of the cisternae. The forming face of the Golgi apparatus is associated with large irregular-shaped membrane profiles which are apparently continuous with the endoplasmic reticulum since scattered groups of ribosomes are sometimes found on the membrane of these structures (Fig. 5). Dense bodies, which probably represent autophagic lysosomes are a regular feature of these cells (Fig. 6). They are up to 1 μ in dia. and occur in any part of the cytoplasm. Internally, they consist of electron-transparent units and multilaminated units both of which are set in an electron-dense matrix. The nuclei have sparsely scattered chromatin and possess one or two nucleoli (Fig. 7). The nuclear membrane is usually irregular in outline and has numerous nuclear pores (Figs. 4, 7).

Each neurosecretory cell is separated from adjacent nerve cells by one or two layers of glial cell process. This glial cell layer varies from 100 mμ to 1 μ in thickness. The axons of the neurosecretory cells are about 1 μ in dia. and pass between the cell bodies as they run towards the interior of the optic lobe (Figs. 3, 7). A short distance from the cell bodies the axons become intermingled with another type of axon containing small electron-dense neurosecretory granules about 700 nm in dia. (57 to 93 nm range). Synapses of these latter axons onto the axons containing the large neurosecretory granules have been found (Figs. 8, 9). Groups of synaptic vesicles (44 nm mean diameter, 37–56 nm range) occur in the presynaptic axon, often intermingled with the electron-dense granules. The thickened membranes of the synaptic junction are parallel and are spaced about 20 nm apart. There is a plate of dense material in the synaptic cleft and a sub-synaptic web is always present at each synapse.

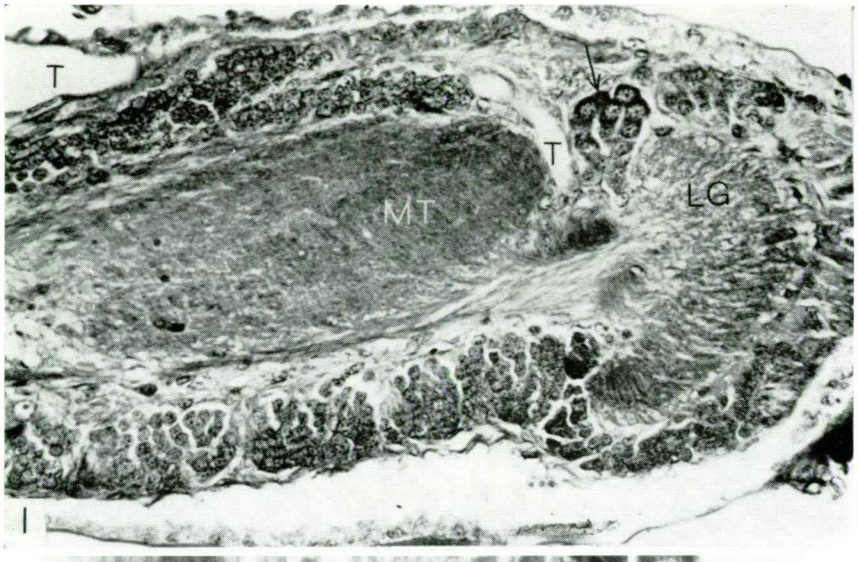
After passing through the synaptic region, the neurosecretory axons cross over to the other side of the optic lobe at the distal optic chiasma and run towards the brain. The bundle of neurosecretory axons in this region is about 10 μ in dia. Numerous neurotubules are present in the axons and the neurosecretory granules tend to occupy the peripheral portions of the axoplasm (Fig. 10). Throughout the axoplasm of both types of neurosecretory fibres there exists a ramifying system of tubular endoplasmic reticulum (Figs. 8–10). The diameter of the tubules varies from 40 to 60 nm. This is considerably larger than the diameter of the neurotubules (20–22 nm) and there appears to be no continuity between these two structures as suggested by VOLLRATH (1969) for neurosecretory axons in rats. Branches of this system often appear to be associated with mitochondria and neurosecretory granules. This association may be merely fortuitous or perhaps represents a functional configuration. In the synaptic regions, this tubular system is present (Figs. 8, 9), but does not appear to give rise to synaptic vesicles as suggested by VOLLRATH (1969).

At the proximal optic chiasma, the bundle of neurosecretory axons once more cross over and continue towards the brain. The fate of these axons in the brain has not been investigated at this stage.

DISCUSSION

The neurosecretory cells in the optic lobe of *P. americana* are yet another example of neurosecretory cells which do not stain with paraldehyde fuchsin, but remain acidophilic after permanganate oxidation. The variable staining reaction with the azan technique may be due to some cyclical activity of the cells but may also be caused by slight variations in the staining technique. The red staining by azocarmine is the more usual situation in acidophilic C-cells of insects (e.g. RAABE, 1965). The affinity of NSM for the aniline blue counterstain has not been reported in insects except for the violet aggregates in the perisympathetic neurohaemal organs of the ventral nerve cord in phasmids (RAABE, 1965).

The contention of BRADY and MADDRELL (1967), that acidophilic NSM of C-cells corresponds with electron-transparent granules and that paraldehyde



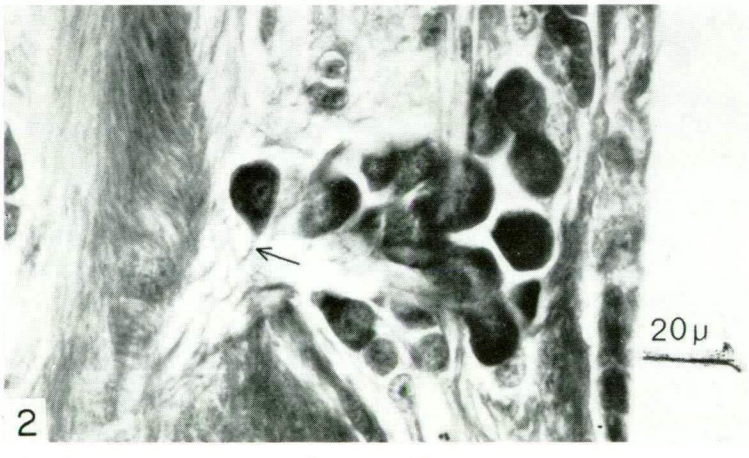


FIG. 1. Horizontal section of the optic lobe showing the neurosecretory cells (arrow) in relation to the medulla terminalis (MT), lamina ganglionaris (LG), and the main trachea (T). Azan technique.

FIG. 2. Neurosecretory cells in the optic lobe. An axon (arrow) can be seen running towards the interior of the optic lobe. Azan technique.

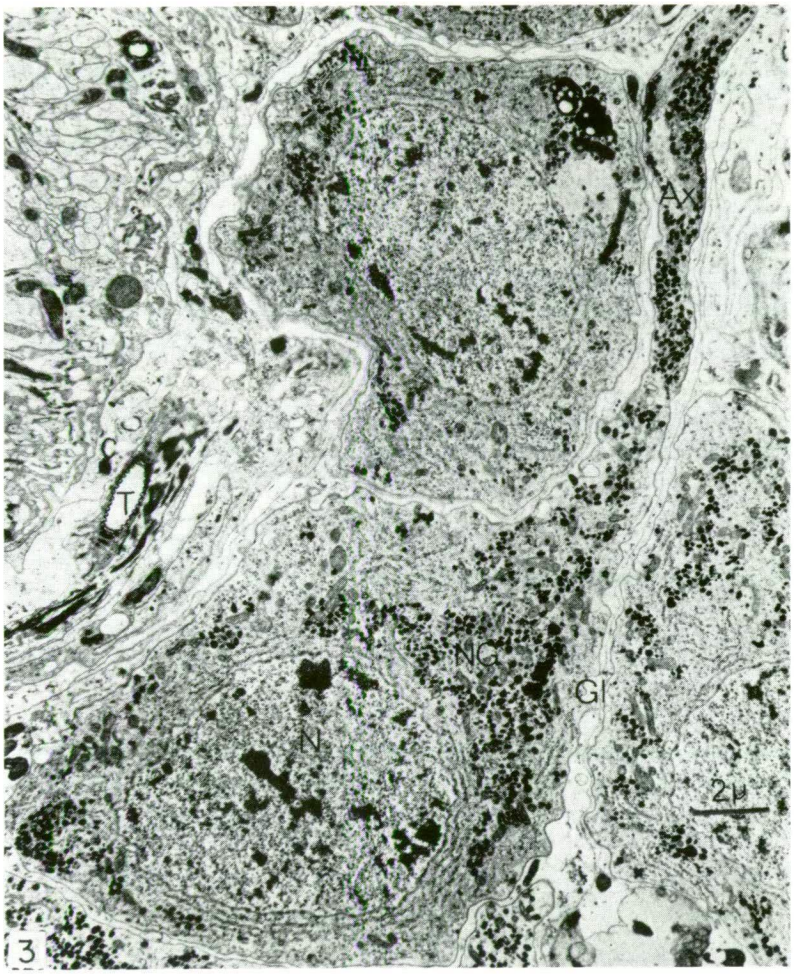


FIG. 3. Electronmicrograph showing numerous neurosecretory granules (NG) in the cell bodies. The axon (Ax) of one of these cells can be seen running between the other cells. Each nerve cell is surrounded by glial tissue (Gl). Trachea (T) are usually present close to the neurosecretory cells.

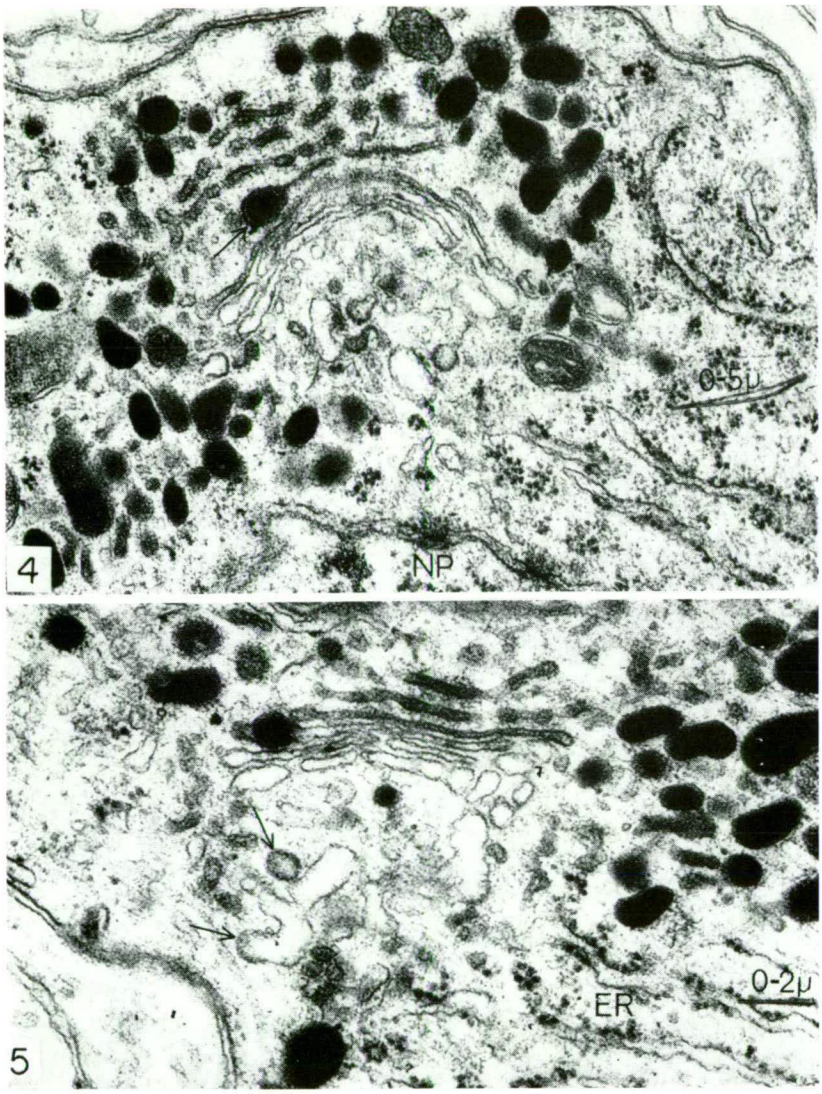


FIG. 4. The Golgi bodies show a marked polarization in their structure. A neurosecretory granule (arrow) can be seen forming at the periphery of the Golgi body. A nuclear pore (NP) is present in the nuclear membrane.

FIG. 5. Another Golgi body showing the apparent continuity between it and the endoplasmic reticulum (ER). Finger-like projections (arrows) from irregular-shaped profiles show an accumulation of dense material.

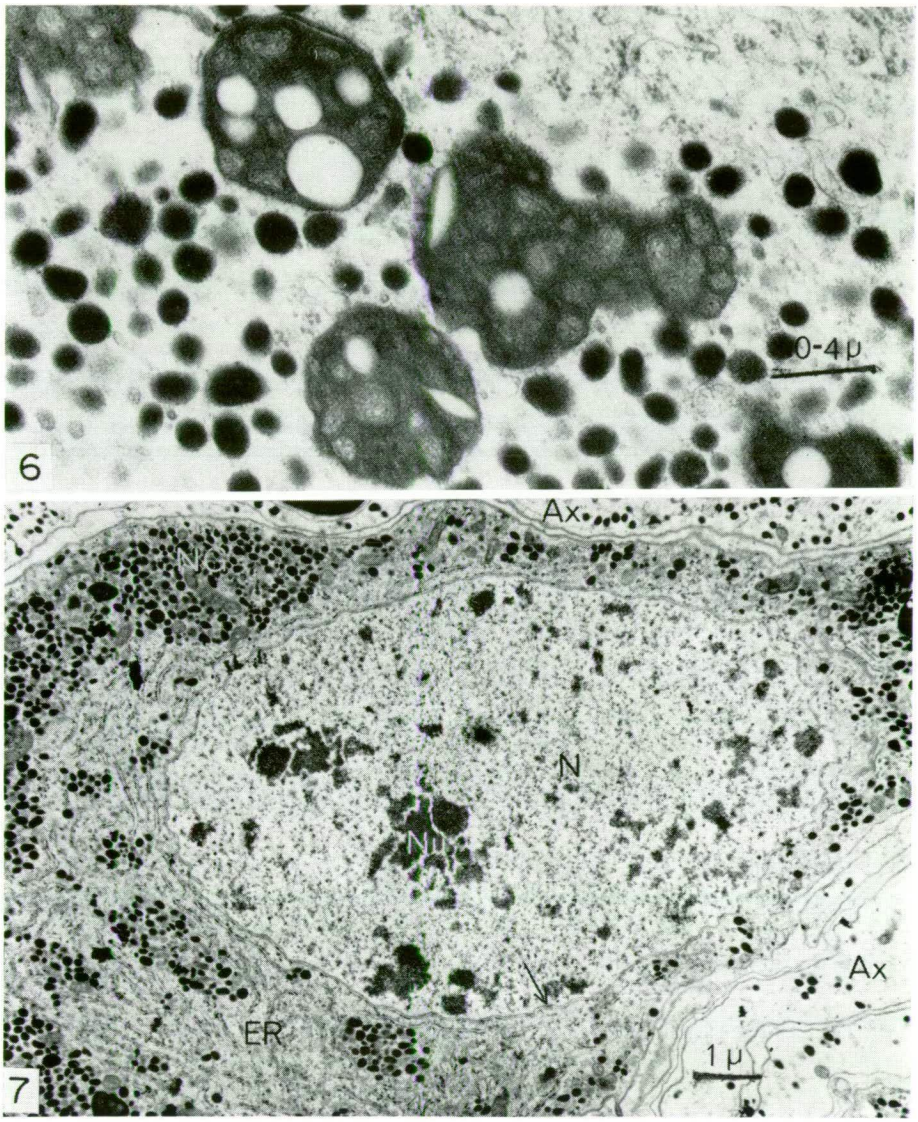


FIG. 6. A group of dense bodies which are possibly autophagic lysosomes. Each body is made up of electron–translucent units and multilaminated units.

FIG. 7. A neurosecretory cell body showing nucleolar material (Nu) within the nucleus (N). A nuclear pore is indicated by the arrow. The endoplasmic reticulum (ER) is fairly well developed and numerous neurosecretory granules (NG) are present. Several axons (Ax) from other neurosecretory cells can be seen passing by this cell.

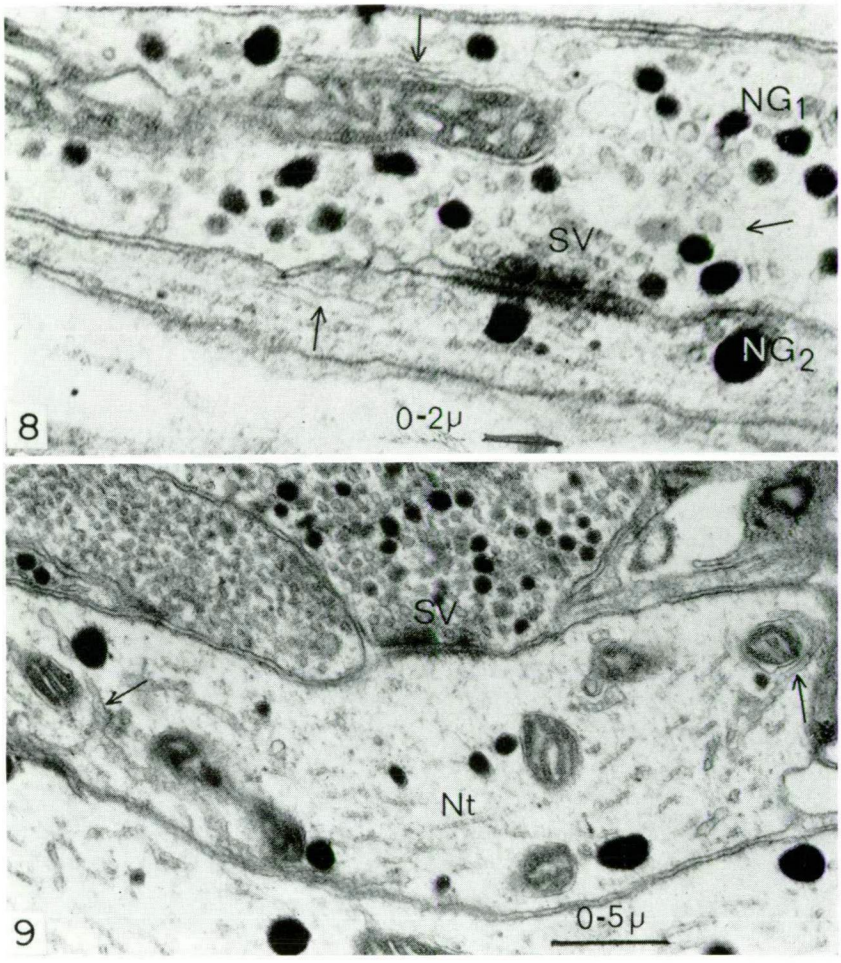


FIG. 8. Electronmicrograph of a synapse between an axon containing small neurosecretory granules (NG_1) and large neurosecretory granules (NG_2). Synaptic vesicles (SV) are present on the pre-synaptic side of the synapse. Tubular endoplasmic reticulum (arrows) is present in both pre- and post-synaptic axons and is often associated with mitochondria (M).

FIG. 9. Similar to Fig. 8. Neurotubules (Nt) and branching tubular endoplasmic reticulum (arrows) are present in the post-synaptic axon. SV, synaptic vesicles.

FIG. 10. The bundle of neurosecretory axons which run back towards the brain. The neurosecretory granules (NG) tend to occupy the peripheral parts while neurotubules (Nt) and tubular endoplasmic reticulum (arrows) occupy the central portions.

fuchsin positive NSM corresponds with electron-dense granules does not hold for the NSM in the cells in the optic lobe of the cockroach. In this case acidophilia occurs with electron-dense granules 100 to 170 nm in dia.

The variable amounts of NSM stainable with the azan technique may indicate different stages in the secretory cycle or that the cells are a heterogenous group of cell types. It is most probable that the first alternative is correct since all the cells stain the same colour and show variations in staining intensity and not variations in type of staining reaction in any one animal. The histochemical test for arginine is interesting in this respect in that the nuclei of strongly positive cells are very granular and the more weakly staining cells have almost clear nuclei when viewed with phase contrast optics. The granular nuclei appear to be associated with actively synthesizing cells whereas clear nuclei appear to be associated with less active or inactive cells. This may again indicate some sort of cyclical activity in these neurosecretory cells. The study of cyclical activity in these cells is being investigated further.

There has been several histochemical studies on acidophilic neurosecretory cells in insects. In the C-cells of the phasmid, *Clitumnus extradentatus*, the NSM is a protein rich in tryptophan (RAABE and MONJO, 1970). BAUDRY and BAEHR (1970) working on *Rhodnius*, found that the protocerebral and suboesophageal C-cells are positive for tryptophan but not the C-cells of the ventral nerve cord. The NSM of the neurosecretory cells in the optic lobe of *P. americana* is likewise a protein rich in tryptophan but also contains considerable amounts of the basic amino acid, arginine. This latter fact probably accounts for the histological staining reaction of the NSM.

At the ultrastructural level, this group of cells show all the characteristics of secretory neurones. The well developed endoplasmic reticulum, the prominent Golgi bodies and the accumulated masses of elementary neurosecretory granules indicate that the cells are synthetically active. The increased electron density of the more mature cisternae of the Golgi apparatus indicate that the contents are in a more condensed state and the cells are actively producing NSM (SCHARRER and BROWN, 1961). The apparent continuity between the endoplasmic reticulum and the Golgi apparatus is supported by similar observations on other tissues (e.g. MOORE *et al.*, 1970). The secretory material appears to be enclosed first within the cisternae of the endoplasmic reticulum, then the membranes of the Golgi apparatus, and finally within the granule membranes (see BEAMS and KESSEL, 1968). At every stage the proteinaceous material is contained within the same membrane, but it is probably modified during transition.

The specialized contact zones between the two types of neurosecretory axons appear to represent true synapses in all morphological characteristics. Synapses between small granule axons (type B fibres) and large granule axons (type A fibres) may indicate the control of neurosecretory axons by aminergic axons. Similar types of synapses have been reported from other insect species and probably represent true synapses, whereas other 'synaptoid' junctions probably represent release sites for NSM (SCHARRER, 1968 for discussion). These synapses

could be the control site for either the regulation of synthesis of NSM in the perikaryon, or the transport of NSM down the axons, or the release of NSM material from the axons, or a combination of all or any of these. The perikarya of the cells containing the small granules have not been found so it is impossible to say from where this group of neurosecretory cells is controlled.

These acidophilic cells in the optic lobe constitute a new group of neurosecretory cells in insects. At the moment their function can only be speculated upon, but from their location it would appear that they may be somehow connected with the eyes and respond to changes in the light intensity or photoperiod. The physiological clock controlling activity rhythms has been located in the optic lobes of *Periplaneta* (review by BRADY, 1969), and these cells may well be associated with this clock mechanism.

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